Sec61 mediates antigen translocation into the cytosol for cross-presentation

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1 Introduction

The immune system is one of the most complex and controlled systems of an organism. It is necessary for the defense against many different threats like bacteria, viruses or fungi. Additionally, it plays an important role during the identification and abatement of cancer. In general the immune system can be divided into two different parts [1].

The innate immune system The first part is called the innate immune system, also known as the nonspecific immune system. It consists of various mechanisms passively protecting against invaders. Additionally, it recognizes structures, which are only present on pathogens.
The passive mechanisms of the innate immune system include all epithelial surfaces building a mechanical barrier for pathogens as well as the mucus in the respiratory tract, which can capture invaders and transport them out of the organism. The second, more active group of mechanisms depends on the recognition of highly conserved pathogen associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS). These structures can be detected by pattern recognition receptors (PRR). One example for PRRs are toll like receptors (TLRs), which are expressed on different cell types, like neutrophils or dendritic cells (DCs). After ligand-detection they are able to activate multiple signaling pathways resulting in the modification of cellular functions and thereby in the activation of different immune mechanisms of these cells.
The combination of these protection structures of the innate immune system enables a very fast recognition of invaders and represents the first line of defense against an infection [2, 3].

The adaptive immune system The second part of the immune system is the adaptive immune response building upon the innate system [4]. This second line of defense is only present in vertebrates and provides a more specific and longer lasting protection. After a short activation period, it offers a diversified repertoire of mechanisms to delete the pathogen and eliminate the threat to an organism. In addition to its high specificity, this part of the immune system also contains a memory of previously encountered pathogens, which enables a very fast reaction in the case of reinfections [5, 6].

The adaptive immune system is again divided into two parts, the humoral and the cellular immune response. The humoral response is mediated by antibodies, which are highly specific to a particular pathogen. They are produced by B-cells with the assistance of $CD4^+$ T-helper lymphocytes (T-helper cells) and designed to protect against extracellular pathogens [1, 7].
The other part of the adaptive immune system is called the cellular immune response, which is specialized to remove intracellular pathogens like viruses. To enable this cellular response nearly all cells of an organism present their intracellular peptides at their surface. After activation, $CD8^+$ cytotoxic T-lymphocytes (CTLs) recognize presented peptides of pathogens (like parts of viruses) or other unknown proteins (for example from cancer cells) and eliminate the infected or mutated tissue [8, 9, 10].

1.1 Dendritic cells in the immune system

Dendritic cells (DCs) belong, besides macrophages (Mϕ), B-cells and some other cell types, to the group of professional antigen presenting cells (APCs) [1]. These APCs have the ability to take up various antigens, process them and present their peptides at their cell surface to activate other parts of the adaptive immune system. While B-cells internalize antigens with a high specificity by B-cell receptors [11], DCs and Mϕ have different possibilities to pick up antigens. They can nonspecifically internalize soluble antigens from their surrounding by pinocytosis, particles by phagocytosis or alternatively use various receptors to take up defined substances, including pathogens, by receptor-mediated endocytosis [12, 13, 14]. Macrophages mainly fulfill regional functions in the peripheral tissue, like the local elimination of pathogens or uptake of dead cells (for example cells killed by CTLs). In contrast to this, DCs build a bridge between the innate and adaptive immune system and play a very important role during the activation of adaptive immune responses [14, 15, 16, 17].
The DCs of an organism are a widespread system of cell-subtypes with different functions [18]. Roughly they can be divided into lymphoid-resident DCs, migratory DCs and inflammatory DCs [1, 19]. Lymphoid-resident DCs differentiate from bone marrow-derived myeloid precursor cells and are resident in lymphoid tissues, like the lymph nodes or the spleen, where they represent the main part of the DC population [20, 21]. They again can be split into different subsets specified by CD4 and CD8 expression. These DC subsets differ in their ability to process antigens as well as in their presentation mechanisms. $CD8^+$ DCs are described to be able to efficiently present extracellular antigens on their cell surface to CTLs, while the $CD4^+$ DCs and double negative DCs prefer the activation of CTLs by the presentation of intercellular antigens [22]. In contrast to lymphoid resident DCs, inflammatory DCs, also called monocyte-derived DCs, differentiate from monocytes. This subset is activated in presence of stimulatory signals and is recruited to the site of inflammation. There, they support the local immune response

of macrophages and migratory DCs [23, 24]. Migratory DCs, again differentiating from bone marrow-derived precursor cells, are present in many peripheral organs in an inactive state [25, 26]. Also here subgroups differing in their possibility to present antigens are described. For these, CD103 is used to distinguish the subpopulations [27, 28], because CD4 and CD8, which are available for the characterization of lymphoid-resident DCs, are poorly expressed on migratory DCs. The presence of CD103 is thereby associated with the activation of CTLs by the presentation of extracellular antigen. Recently an additional marker of DCs, which mediate the presentation of extracellular antigens to CTLs, was discovered (XCR1) [29]. Present on migratory DCs as well as lymphoid-resident DCs, this marker reveals a connection of these two subgroups and enables a further characterization of the DC-subsets in the organism [30].
In the peripheral tissue, migratory DCs are able to take up big amounts of molecules and particles from their surrounding to screen for pathogens. After uptake of an antigen and activation by innate PRR like TLRs, these DCs start to mature [4, 16] and up-regulate different sets of chemokine receptors, which induce and control their migration towards the lymphoid tissue [31, 32]. During maturation and movement, co-stimulatory molecules like CD40 and CD80/86 are up-regulated [16, 33] and proinflammatory cytokines secreted. Additionally, the antigen processing is enhanced. This increases the number of presented antigens on the surface of the migrating DC. In contrast to this, the antigen uptake is severely reduced [4, 16]. After arriving in the lymphoid tissue, the antigens are displayed to T-lymphocytes. The specific recognition of the presented antigen in combination with co-stimulatory molecules as well as inflammatory cytokines finally leads to an efficient activation of the effector function of T-lymphocytes. Depending on the mode of antigen presentation by the DCs, different subsets of T-lymphocytes are activated [8].

1.2 Different pathways of antigen processing and presentation

DCs as well as other professional APCs have two major possibilities to present protein-derived antigens to activate different types of T-lymphocytes. On the one hand, they can present intracellular antigens on major histocompatibility (MHC)-I molecules. This enables the activation of CTLs, which have a specific receptor directed against the MHC-I peptide complex on the DCs. After stimulation, these T-cells can scan cells in the periphery for the presentation of the same MHC-I peptide complex. Because all nucleolus-containing cells express MHC-I molecules and present intracellular peptides to their surrounding, CTLs can screen the whole body for potential intracellular infections and destroy contaminated cells [8, 34, 35]. On the other hand, professional APCs can display peptides on MHC-II molecules. These MHC-II complexes are loaded with antigens, which have been taken up from the surrounding of the APC. Afterwards, this complex can be recognized by T-helper

cells, which are stimulated, when the T-cell receptor matches to the presented MHC-II peptide combination. Subsequently, activated T-helper cells can for example support the antibody production of B-cells against the pathogen, which has been detected by the T-helper cell before [8].

1.2.1 MHC-I presentation

The presentation on MHC-I molecules is a constant mechanism in all nucleated cells and used to detect changes in the protein expression of a cell, for example caused by viruses or bacteria. Therefore, intracellular proteins are labeled for degradation (mostly by polyubiquitination) and segmented into small peptides by cytosolic proteasomes. These fragments can afterwards be loaded on MHC-I molecules for presentation [34, 36].
Generally, proteasomes are used by cells to degrade old or misfolded proteins. They consist of four subunit complexes in combination with a regulatory structure on each side. The two subunits in the middle mediate the proteolytic activity by N-terminal threonine residues [37, 38, 39]. In professional APCs a second set of subunits for the active region of the proteasome is available, namely lmp2 (low molecular mass protein 2), lmp7 and MECL-1 (multi-catalytic endopeptidase complex like I). These are produced and integrated into proteasomes in parallel to the normal proteasomal subunits and their expression is increased after activation of the DC by pathogens or occurrence of proinflammatory cytokines [40, 41]. The replacement of the normal subunits by the immune-induced components leads to a changed structure inside of the active center, which influences the speed of protein degradation as well as the preferred cutting sides [42, 43]. The proteasomes containing these subunits are called immunoproteasomes and described to be important for the generation of "immunogenic" peptides, which can be presented more efficiently on MHC-I molecules at the surface of APCs [44]. After proteasomal degradation of the

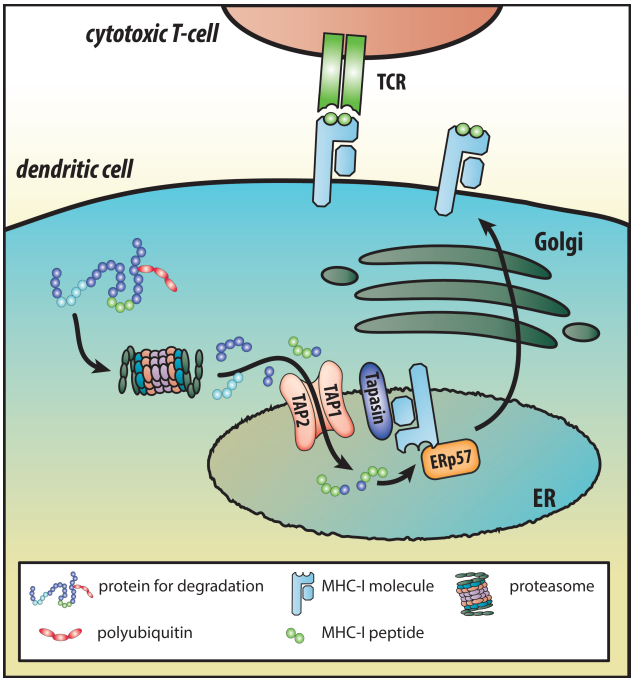

Figure 1.1: ***The MHC-I presentation pathway*** After proteasomal degradation, peptides are transported into the ER and loaded onto MHC-I molecules, which subsequently can be presented to CTLs on the cell surface.

protein, the resulting 6 to 30 amino acids long peptides are transported into the endoplasmatic reticulum (ER) via a transporter named TAP (transporter associated with antigen processing). The TAP complex consists of two different subunits (TAP1 and TAP2) [45, 46] and interacts at its luminal side via tapasin with unloaded MHC-I molecules, which contain a heavy and a light chain (β2-micro-globulin). Furthermore, this complex is stabilized by different chaperones like calreticulin, calnexin as well as Erp57. After the peptides are imported into the ER, they can be loaded on MHC-I molecules to form the presentation complex [47, 48]. Finally, to completely fit the peptide into the binding site of the MHC-I molecule the aminopeptidase ERAP (ER-associated peptidase) is required to split overhanging amino acids during the loading procedure [49].

1.2.2 MHC-II presentation

In contrast to intracellular antigens, extracellular antigens are presented on MHC-II molecules, which are mainly expressed by APCs [1, 50]. Therefore, pathogens are taken up by various mechanisms like pinocytosis, phagocytosis or receptor-mediated endocytosis and are transported into late endosomal and lysosomal compartments. In these, the proteins, for example from bacteria, are cleaved by several proteases (e.g. different cathepsins) as well as other digestive enzymes (e.g. phosphatases or nucleases) [51, 52]. This process provides the peptides for the loading of MHC-II molecules.

The MHC-II complex itself consists of two transmembrane chains, is produced in the ER and stabilized by the binding to an invariant chain (Ii). Additionally, the Ii blocks the peptide binding site to avoid the loading with ER peptides [53]. After synthesis, this complex is transported through the golgi-apparatus to late endosomal compartments, where the digested peptides are present.

The compartment that is thereby generated is described as a class II vesicle (CIIV). Inside of this compartment the Ii is digested by proteases and only a small fragment (CLIP, Class II associ-

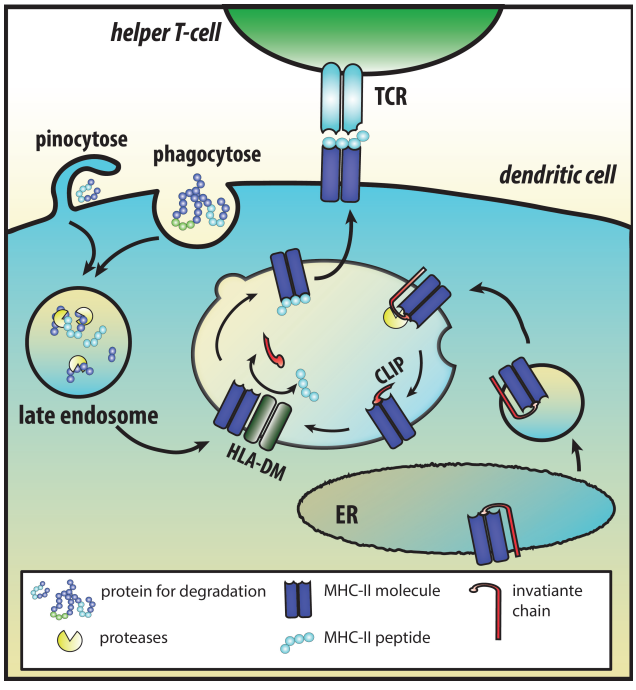

Figure 1.2: ***The MHC-II presentation pathway*** The extracellular antigen is taken up and digested in lysosomal compartments. The resulting peptides are loaded on MHC-II molecules, transported to the surface and presented to T-helper cells.

ated invariant chain peptides) remains in the peptide binding site of the MHC-II molecule. For antigen presentation, the CLIP can now be exchanged with antigen peptides by the MHC-like molecule HLA-DM [54]. Sequentially, the loaded MHC-II peptide complex is transported to the cell surface and presented to T-helper cells.

1.3 Cross-presentation as additional presentation pathway

A third possibility for DCs to present antigens to T-cells is cross-presentation [55, 56]. This allows the immune system to display extracellular proteins on MHC-I molecules and is important for the immune response against pathogens that impair direct presentation of antigens or do not infect APCs [57, 58], for example papilloma viruses which are mainly infecting epithelial cells [59]. In addition, tumor cell-fragments can be taken up by APCs and subsequently be cross-presented to enable an immune response against the tumor [60, 61]. Although cross-presentation is reported for several phagocytic cells, DCs are considered to be the primary cross-presenting cells. However, despite of intensive investigations, the molecular mechanisms behind the cross-presentation pathway are still not fully clarified. Nonetheless, the various mechanisms described during the last years point out that not only one single possibility for cross-presentation exists. So far, at least four different pathways are known and discussed.

1.3.1 The “endosome to cytosol” pathway

The most prominent pathway for cross-presentation is the “endosome to cytosol” pathway (Fig. 1.3) [62, 63, 64], which is also called “phagosome to cytosol” pathway for particulate antigens [65, 64]. During this presentation pathway, the antigen is taken up by the DC and transported into a specialized compartment, where the cross-presentation is mediated. From this the antigen is translocated into the cytosol to enable proteasomal degradation. The peptides generated thereby are afterwards reimported into these specialized compartments [66, 64, 67, 63] or transported into the ER [65, 68, 63]. There, the peptides are loaded on MHC-I molecules, which can be presented on the cell surface to CTLs.

Protection of the antigens from lysosomal degradation To enable a constant supply of antigens and thereby a cross-presentation over a long period of time, the maturation of the antigen-containing compartment needs to be slowed down to avoid lysosomal degradation, which would lead to a rapid destruction of antigenic epitopes [62, 69, 70, 71]. Besides low expression of lysosomal proteases in DCs in general [72], the pH in these compartments is stable at a neutral level to keep pH-dependent lysosomal proteases inactive. These conditions are mediated by the recruitment of the NADPH oxidase NOX2 [73, 74], which produces reactive oxygen species to neutralize protons within the compartment. Additionally, lipid bodies controlled by the interferon-inducible ER-resident GTPase (Igtp), are involved in lowering down the maturation of the antigen-containing endosomes [75]. However, the exact mechanisms of this influence by lipid bodies is not clarified so far.

Since not all endosomes are slowed down in their acidification and some mature towards degradative lysosomal compartments, it is important for cross-presentation that the antigen is localized in a stable endosomal compartment after its uptake. At least for soluble antigens the decision, whether an antigen is transported into a slow-maturing endosome or is directed towards lysosomal degradation, which can mediate MHC-II presentation, is already influenced by the used uptake mechanism and the involved receptors. While receptors like the mannose receptor [69] or monovalent dectin1 [76] direct their ligands into a cross-presenting compartments, pinocytosed antigens or ligands taken up by other receptors, like scavenger receptors [69] and cross-linked dectin1 [77], prefer the transport towards lysosomal compartments and MHC-II presentation. The dectin1 receptor additionally shows that not only the receptor itself, but also the structure of the ligand (for dectin1 it is β-glucan) has an influence on the antigen localization and its fate. While monovalent β-glucan is mainly transported into stable endosomal compartments and therefore is cross-presented, multivalent β-glucan rather ends up in degradative lysosomal compartments and is available for presentation on MHC-II molecules.

Antigen processing and loading on MHC-I molecules After antigen uptake and transport into a stable endosomal compartment, a decisive, but still not fully clarified step of the endosome to cytosol pathway is the translocation of the antigen out of the endosomal compartment into the cytosol for further processing.
The needed energy to overcome the membrane barrier between the endosome and the cytosol might be delivered by the ATPase p97, which could act as a driving force for the antigen [78, 66]. It has thereby been shown that the receptor involved in antigen uptake might also support the antigen translocation by recruiting the ATPase p97 via ubiquitination to the site of the antigen-containing endosome [66]. Additionally, it has been reported that some proteins need to be unfolded for their export into the cytosol and that the disulfide bonds of the protein have to be split by the γ-interferon induced lysosomal thiol reductase (GILT) [79]. Arriving in the cytosol, the exported antigen is delivered to proteasomes, where it is efficiently cleaved into immunogenic peptides [44, 80].

After degradation, these peptides are reimported by TAP into the ER [63, 65, 68] or into the antigen-containing compartment itself, which has recruited the MHC-I loading machinery before [63, 64, 66, 67]. In these compartments the processed peptides are loaded on MHC-I molecules and trimmed by different aminopeptidases, like ERAP in the ER or the endosomal IRAP, which is more efficient at the neutral pH, in the endosomal cross-presenting compartments [63, 65, 81]. Although the ER as well as the endosomal compartments are described as locations for MHC-I loading, recent data suggest that antigen loading for cross-presentation might mainly occur within the antigen-containing compartment rather than in the ER [62, 67].

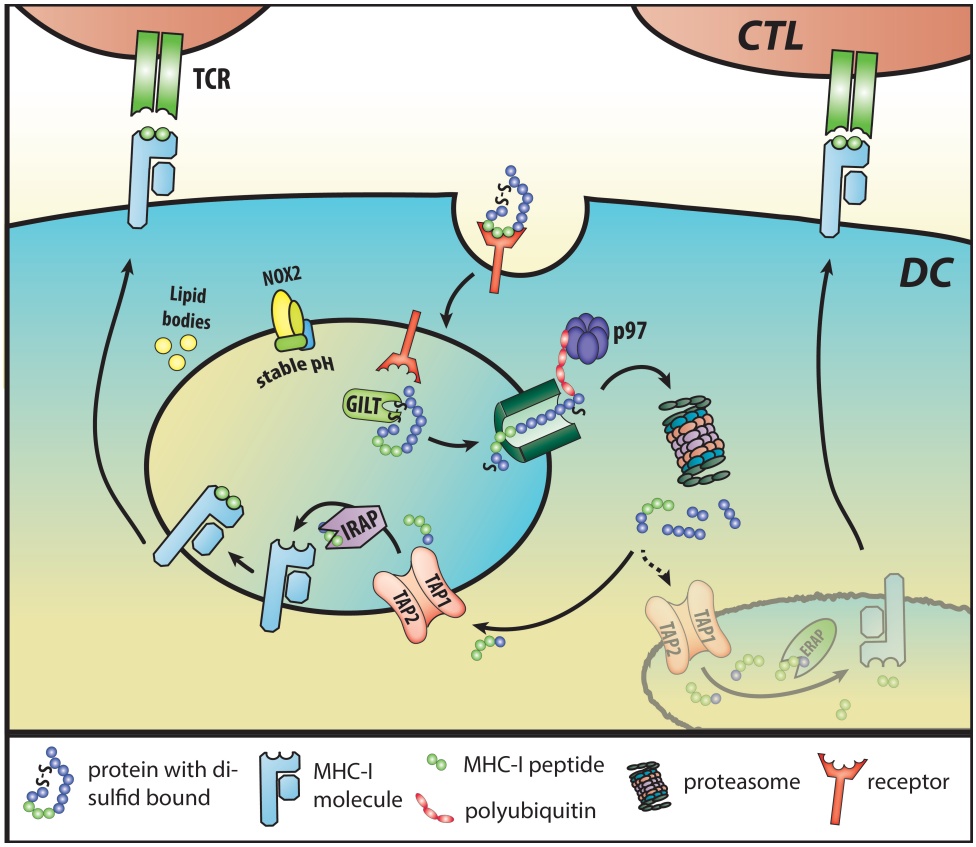

Figure 1.3: ***The "endosome to cytosol" pathway of cross-presentation*** Extracellular antigens are taken up by DCs and transported into a specialized compartment for antigen cross-presentation. The antigens are exported into the cytosol, degraded by proteasomes and the peptides are reimported into the same compartment or alternatively into the ER. There they are loaded on MHC-I molecules for presentation to CTLs.

Recruitment mechanisms of components for cross-presentation towards antigen-containing endosomes Although the presence of many components of the cross-presentation machinery has been shown on these specialized endosomal compartments, it is not fully understood how the recruitment of the needed ER-components for the loading machinery works. However, some parts of potential pathways have been uncovered during the last years. Besides the possibility of direct ER-involvement in endocytosis by providing membrane for antigen engulfment [82], especially the role of different rab (ras-related in brain) and SNARE proteins (soluble N-ethylmaleimide-sensitive-factor attachment receptor) has been described in several publications. These two protein families are important for vesicular fusion events and are therefore able to mediate and control different recruitment processes [83]. In context of NOX2, which is important for pH regulation in the cross-presenting endosomes, it has been shown that its recruitment depends on rab27, a GTPase described to be involved in exocytosis of secretory vesicles [73, 84]. Also the rac2 GTPase as well as VAMP-8, interacting with the partly phagosomal SNARE proteins syntaxin4 and SNAP23, were reported to be important for NOX2 translocation to antigen-containing compartments [63, 85]. During the recruitment of the endosomal peptidase IRAP a co-localization with rab14 as well as with the SNARE protein syntaxin6 was observed, indicating that these two factors might also contribute to the recruitment machinery [63, 86]. Additionally, the SNARE protein sec22b seems to play a central role for the translocation of parts of cross-presentation machinery (like TAP and Tapasin) from the ER to the endosomal cross-presenting compartments [64].
Besides the molecular mechanism of the recruitment pathways also the origin of the single components of the cross-presentation machinery in endosomal compartments is under intense investigation. In a recent publication it was described that the MHC-I molecules for cross-presentation are recruited rather from the plasma membrane than from the ER. Therefore the MHC-I molecules are transported from the plasma membrane to a sort of “storage” compartment, which afterwards provides MHC-I molecules exclusively for cross-presentation and is essential for efficient presentation of extracellular antigens on MHC-I molecules [67]. Interestingly, the supply with MHC-I molecules is completely independent from the sec22b-mediated protein transport [67], indicating that several, more or less independent, pathways are needed for the full function of the cross-presenting endosomal compartment.

1.3.2 The vacuolar pathway

The second mechanism of cross-presentation is the vacuolar pathway (Fig. 1.4 A). The antigen is thereby taken up and directly digested in endosomal or lysosomal compartments by proteases like cathepsin S [87, 88]. Afterwards, generated fragments are loaded on MHC-I molecules inside of these compartments and can be presented on the cell surface later on.

The fact that MHC-I molecules can traffic to different compartments for peptide loading has already been postulated before [89], but the origin of the MHC-I molecules for endosomal antigen loading is so far not fully clarified. Newly synthesized MHC-I molecules might be trafficked from the ER to antigen-containing compartments. Alternatively, MHC-I molecules from the cell surface could be reused and the old peptides replaced by new ones. This exchange might be supported by the acidic environment inside of the endo-lysosomal compartments [90]. Important for the supply with MHC-I molecules is thereby a conserved tyrosine residue in the cytoplasmatic tail included in the heavy chain of the MHC-I molecule. The lack of this amino acid impairs the ability to present extracellular antigens to CTLs [91]. As this cross-presentation mechanism takes place within a single compartment, it is also independent of any proteasome or TAP activity.
In the context of MHC-I loading in endo-lysosomal compartments, also the possibility to "cross-present" peptides engaged by autophagy is described. Especially in DCs, cytosolic aggregates of mainly ubiquitinated proteins (DC aggresome-like structures, DALIS) are enclosed by autophagy and degraded in endo-lysosomal compartments. Subsequently, the resulting peptides can be presented on MHC-I molecules [92].

1.3.3 Gap-junctions

Apart from of the endosome to cytosol and the vacuolar pathway for presentation of extracellular antigens on MHC-I molecules, two additional pathways for cross-presentation are described in the literature, which may play a role under certain conditions.
The first one is utilizing gap-junctions to transfer antigens between two cells and to couple their peptide presentation (Fig. 1.4B). Gap-junctions consist of connexins and build a channel between two interacting cells to connect their cytosol. Through this construct the already processed peptide is transported from one cell to another (for example from a viral infected cell to a DC) [93]. Afterwards, the peptide directly enters the ER via the TAP channel and is loaded on MHC-I molecules by the same way as an endogenous peptide [94]. This cross-presentation pathway might be used to detect viruses, which are hiding in cells and suppress their direct presentation. The transfer of virus-peptides to DCs by gap-junctions might thereby enable an immune response. The theory that this system plays a role during viral infection is enforced by the fact that for example herpesviruses can suppress the expression of connexins [93]. Additionally, gap-junctions can be involved in the immune defense in the surroundings of tumor cells. Investigations have shown that *Salmonella*-infected tumor cells increase the formation of gap-junctions. Subsequently, the antigen transfer through these channels to DCs can induce an immune response against the tumor cells. Therefore, this might also be usable for potential tumor therapies in the future [93, 95].

1.3.4 Cross-dressing

The second alternative method for APCs to cross-present an antigen is to take over the MHC-I peptide complexes from other cells, which are infected and have already loaded antigenic peptides on the MHC-I molecules at their cell surface (Fig. 1.4 C). This process is independent of any antigen uptake or processing and enables the DC, which receives the MHC-I peptide complex, to activate CTLs for an efficient immune response [96, 97]. Studies have revealed that a short fusion event of the plasma membranes is essential for the translocation of the MHC-I peptide complex. The involvement of small vesicles like exosomes is still discussed controversially [96, 98].

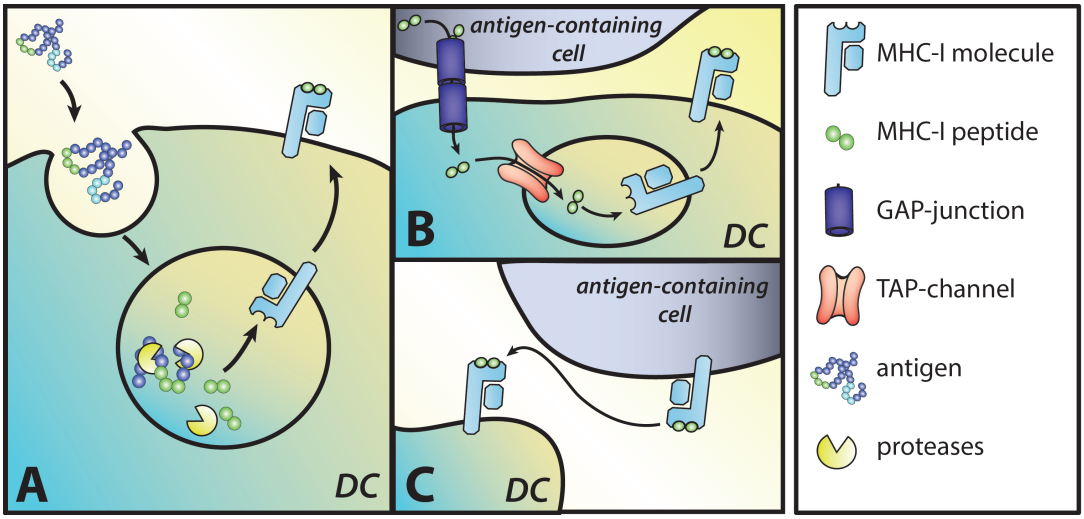

Figure 1.4: ***The vacuolar pathway as well as cross-dressing and gap-junctions in cross-presentation*** **A** The proteasome independent vacuolar pathway. Peptides are generated by intra-endosomal proteases and directly loaded on MHC-I molecules for presentation. **B** The peptides for cross-presentation are transferred to DCs by gap-junctions. **C** Loaded MHC-I molecules are transferred to DCs (also called cross-dressing).

1.4 TLRs and their influence on cross-presentation

Signaling and trafficking of TLRs To activate the immune system, DCs have to be stimulated by PRRs like TLRs [16, 33]. Contacts between PRRs and their ligands thereby influence not only the expression of activation markers, but also intracellular mechanisms like protein interactions [99]. In context of antigen presentation especially TLRs play a central role. Due to their trafficking and recruitment to endosomes or phagosomes, they allow a compartment-specific control for potential ligands as well as a spacial response to their stimuli [100]. An example is the TLR4. It is natively localized at the plasma membrane of the cell and is activated by LPS (lipopolysaccharide). In

the presence of its ligand, it recruits the TIRAP (toll-interleukine 1 receptor (TIR) domain-containing adaptor protein) as well as MyD88 (myeloid differentiation primary-response protein). This recruitment leads to a first response originating from the cell surface. Subsequently, the receptor and its ligand are transported into an endosomal compartment, from which an additional signaling by TRIF (TIR domain-containing adaptor inducing IFN-β) in combination with TRAM (TRIF-related adaptor molecule) occurs. Importantly, this second signaling step strictly depends on the endosomal localization of the TLR4 [101]. Besides endocytosis from the plasma membrane, TLR4 can also be recruited from endosomal recycling compartments (ERC) to antigen-containing structures. This occurs for particular antigens, which contain LPS, in a rab11a and adaptor protein 3 (AP3) dependent manner [102, 103].
In contrast to TLR4, another group of TLRs, like TLR9, is mainly localized in the ER. There it associates with UNC93B, which mediates the trafficking of this TLR. After a stimulation of the cell, TLR9 is selectively recruited to compartments, where its ligand (pathogenic DNA) is present. Importantly, the induction of the recruitment of TLR9 requires no DNA-ligand sensing itself, but a Fc receptor γ-mediated uptake of immunoglobulin (Ig)-DNA complexes [104, 105].

TLRs and cross-presentation How TLRs and their signaling directly influence the molecular mechanisms of cross-presentation is still under intensive investigations. As mentioned above the proteasome subunits are exchanged after the stimulation of the DC took place [40, 41]. In addition to this, especially the recruitment of cross-presenting components from the ER to the endosomal compartments is strictly controlled and depends on the detection of defined signals [106]. It was shown that IRAP, which is needed for peptide trimming in endosomal compartments, only co-localized with the MHC-I loading machinery, when a stimulus with yeast cells occurred [63]. Additionally, the translocation of TAP to antigen-containing compartments depends on TRL4 signaling after a LPS stimulus was detected. For this recruitment only a MyD88 signal is important, while a missing TRIF signal did not influence the TAP co-localization with the antigen. Also for the MHC-I molecule a TLR-dependent translocation to antigen-containing compartments has been described. Therefore, a TLR4-MyD88 signal induces the phosphorylation of the SNARE protein SNAP23, which is localized at the antigen-containing compartment and enables a fusion with MHC-I-containing vesicles. In addition, TRIF signaling seems to participate in cross-presentation in general, but the mechanisms behind this pathway are not clarified so far [62].

1.5 The ERAD machinery

One important, but still sparsely described step in cross-presentation is the antigen export out of the cross-presenting endosomal compartment into the cytosol. However, a very similar process is known from the ER, used to recycle misfolded proteins.

1.5.1 From protein misfolding to degradation at the ER

Nearly one third of the proteins synthesized in a cell are transported or co-translated into the ER, where they are folded and modified in different ways. While some proteins find their functional state within seconds, others need several hours. The whole folding process is very sensitive to stress, faulty protein sequences and many other factors. Therefore, it often results in a misfolded protein. To protect the cell from the aggregation of these faulty proteins within the ER, they need to be exported into the cytosol for proteasomal degradation. This recycling process is mediated by the ER-associated degradation machinery (ERAD; Fig. 1.5) [107, 108].

Recognition of misfolded proteins Multiple factors, which are only partly identified, are involved in the detection of ERAD substrates. For ER quality control and recognition of misfolded proteins, especially ER chaperones and chaperone-like proteins (for example Hsp70s or lectins) play an essential role [109]. An example is the chaperone BiP, which is able to recognize hydrophobic parts of misfolded proteins, to keep them in solution and to recruit these proteins via interaction with other factors, like Herp, towards degradation. This detection system is very important for unglycosylated proteins [110, 111, 112, 113]. Besides recognition of hydrophobic domains, the glycosylation structure, which is added to many proteins after their import into the ER, plays a central role in the ERAD substrate detection. Mostly an N-linked glycan structure, consisting of two N-acetylglucosamines, nine mannoses and three terminal glucose residues, is co-translationally added to the proteins. During the folding process of the protein, de- and re-glycosylations are done to coordinate the interactions with the lektin-like chaperones calnexin and calreticulin [114]. When a protein is not folded within several cycles, mannose residues are trimmed by the ER-mannosidase and EDEM1 (ER-degradation enhancing α-mannosidase-like protein 1). This results in a free α-1,6 mannose residue, which is recognized by a receptor of the ERAD machinery (namely OS-9). Subsequently, this receptor directs the misfolded protein towards the proteasomal degradation complex [115, 116].

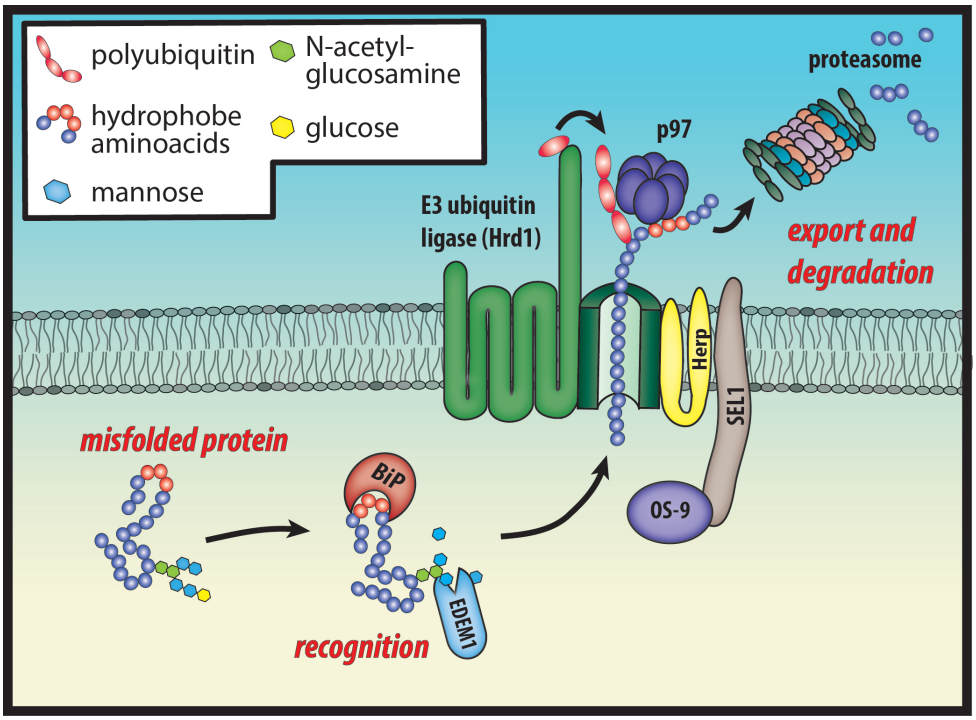

Figure 1.5: ***The function of the ERAD system*** Misfolded proteins are recognized by different chaperones as well as by their modulated glyco-structure. After detection, the targeted protein is transported towards the ERAD complex, where it is translocated into the cytosol. There, it is ubiquitinated and the ATPase p97 is recruited as energy delivering subunit. Finally, the misfolded protein is degraded by proteasomes.

Export and degradation After the detection of the potential ERAD substrate, it is recruited towards the ERAD complex, which consists of substrate detection receptors, several linker-proteins (like SEL1) [117], ER-associated E3 ubiquitin ligases and potential channel proteins for substrate delivery into the cytosol. Also other additional components, like the protein disulfide isomerase (PDI) for splitting disulfide bonds to completely unfold the ERAD substrate and enable the protein translocation, are integrated into this complex [118, 119].

In yeast three different pathways for the ERAD machinery are well characterized. In the center of this process are two ER-associated E3 ubiquitin ligases expressed in yeast. For transmembrane proteins defective on the cytosolic site of the ER (ERAD-C), the E3 ubiquitin ligase Doa10 is described to be important for ubiquitination and further processing. For soluble proteins (ERAD-L) and proteins with defects in their ER-luminal part (ERAD-M) the E3 ubiquitin ligase Hrd1 plays an essential role [120, 121, 122]. Both E3 ubiquitin ligases are multiple transmembrane (TM) proteins with their catalytic domain in the cytosol. At least for Hrd1 it is additionally known that the TM-regions can

recognize misfolded TM-domains of different ERAD substrates itself [123]. In mammals both E3 ligases have orthologs, but additional 14 other ER-associated E3 ligases are identified, emphasizing the complexity of the ERAD machinery [124].
Once the misfolded protein reaches the cytosolic site of the ER-membrane, it is ubiquitinated by the E3 ubiquitin ligase, which marks the protein for proteasomal degradation [125, 126]. At the same time, the ATPase p97, which is described in many cellular mechanisms as the energy delivering component, is recruited directly to the backbone of the exported protein [127]. This interaction is enforced by the polyubiquitin chain, which is linked to the translocated protein [128]. p97 is now proposed to pull the misfolded protein into the cytosol [129, 130, 131]. Additionally, p97 has chaperone-like properties, which keeps the exported protein soluble and thereby mediates the transport to the proteasome, where the final degradation takes place [132, 133]. Interestingly, independent of the selected ERAD pathway, p97 is involved in the final steps of protein degradation for the great majority of ERAD substrates.

1.5.2 Different candidates for the export channel of the ERAD system

One still not fully clarified and intensively discussed step during the ERAD processing is, how the misfolded protein can overcome the membrane barrier for the translocation into the cytosol, where it interacts with p97 and is degraded by proteasomes. For this transport various channel proteins have been described.

1.5.2.1 Hrd1

During the last few years it has been reported that the E3 ubiquitin ligase Hrd1 can directly take over the export of the misfolded protein by building a transmembrane channel by itself or at least contribute to the core of a channel in combination with co-factors [134, 135]. For the translocation of the ERAD substrate the TM-domains of the Hrd1 protein contain several conserved polar and hydrophilic residues. By mutation of these to hydrophobic amino acids the translocation of several substrates is disrupted, while the general interaction of Hrd1 with the misfolded proteins is unaffected [123, 134, 136]. Additionally, it was shown in an *in-vitro* model system that Hrd1 as only membrane component was sufficient to mediate the transport of the ERAD substrate through a membrane structure [135].

1.5.2.2 Derlin1

The mammalian derlin protein family includes at least three different homologs and is member of the rhomboid-like clan of polytopic membrane proteins containing four transmembrane domains [137, 138, 139]. While derlin1 has only a similarity of about 30 % to the sequence of derlin2, derlin2 and derlin3 are closer related to each other (about 70 % of the sequence is identical). The derlin proteins are present inside of ERAD complexes and interact with other components connected to the protein degradation machinery, like ERAD substrate recognition structures and the ubiquitination machinery (including Hrd1). Additionally, derlin1 interacts with p97 and proteasomes via the adaptor protein VIMP [137, 138, 140]. In yeast, derlin is described to be involved in the degradation of luminal ER proteins, but not in the recycling of TM-proteins [141], whereas in mammalian cells all three derlins participate in the translocation of soluble and membrane proteins. The different derlin homologs are thereby responsible for the processing of distinct ERAD substrates. Derlin1 is for example mediating the MHC-I degradation in the presence of the cytomegalovirus (CMV) protein US11 [137, 138]. Under these conditions it was shown that derlin1 interacts with the ERAD substrate on both ER sites, before and after protein export [138]. Additionally, derlin1 was described to be involved in the transport of the pre-pro α-factor [142]. On the other hand, derlin2 and derlin3 are important in the context of an α1-antitrypsin mutation and its ERAD-dependent degradation [143].

1.5.2.3 Sec61

The third channel protein, described in context of the ERAD machinery, is the sec61 protein complex [144, 145]. Sec61, which has first been described and is mainly known from protein synthesis [146, 147, 148], is build of three subunits. The α subunit generates the pore in the membrane, which consists of two transmembrane complexes, each divided in 5 domains. The γ subunit is in close contact to the α subunit and connects their substructures [148]. The importance of sec61 for an organism is emphasized by its high conservation. Compared to *S. cerevisiae* the pore building α subunit of mammalian cells is 55 % identical on protein level [146]. Even more astonishing is the 100 % conservation of the sec61α protein between vertebrates. In addition to this, a second gene encoding a paralogue sec61α protein (sec61α_2) was found in several species [146], which is about 80 % identical to it sec61α_1 and again 100 % conserved between vertebrates. Like sec61α, also the sec61γ subunit shows a high conservation between eukaryotes, while the β subunit, which is required for the lateral release of TM-regions of newly synthesized proteins, is more variable [149].

In context of the ERAD machinery, sec61 interacts with several described ERAD substrates [113, 150, 151, 152, 153]. One example is the cholera-toxin, which is processed in the ER and afterwards misuses the retrograde transport machinery of the ERAD system to get into the cytosol. During the export of the toxin out of the ER into the

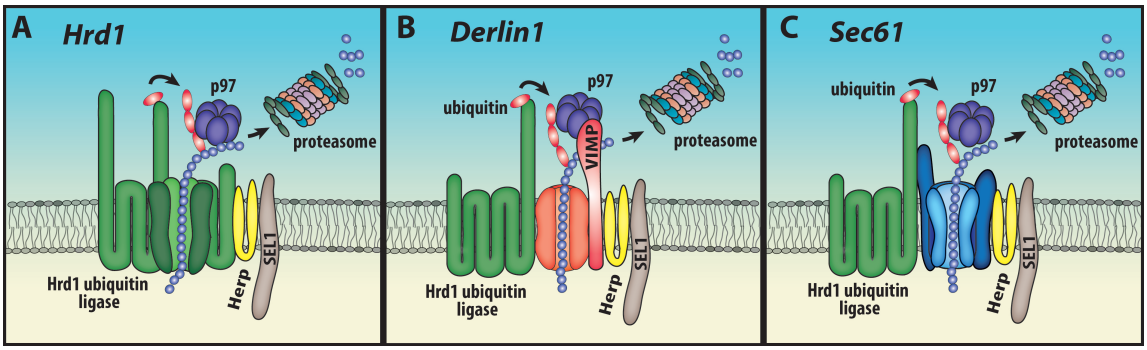

Figure 1.6: ***Models for protein translocation into the cytosol*** **A** The ubiquitin-ligase Hrd1 is mediating the protein export and might create the pore by oligomerization. **B** Derlin1 in combination with Hrd1 builds the channel protein. **C** Sec61 is involved in ERAD and takes over the transport of misfolded proteins.

cytosol it can be co-purified with sec61. Also an interaction of sec61 with SEL1, an important linker protein for the Hrd1 ubiquitin ligase [153] was observed and a contact site between sec61 and the proteasome itself was found [154]. Additionally, two different mutation-constructs of the yeast sec61 protein emphasize its role as channel for the ERAD mechanism. These mutants showed an accumulation of unprocessed ERAD substrates in the ER, while the presence of the cytosolic precursor of the misfolded protein was not increased. This indicates that the protein translocation by sec61 into the ER was unaffected, while the ERAD transport function was impaired [113, 155].

1.5.3 The ERAD machinery in cross-presentation

For some components of the ERAD machinery an important role in cross-presentation is proposed, which might use a similar transport system to deliver antigens from the endosomal compartment into the cytosol for proteasomal degradation. It was shown that p97 is recruited to antigen-containing endosomes and its function is essential for delivering the antigen into the cytosol [78, 66]. However the channel protein for cross-presentation is still not fully identified. Some studies indicate that beside of components of the MHC-I loading machinery, the sec61 channel protein is present in antigen-containing phagosomes [78]. Also the inhibitor exotoxinA, described to interact with sec61 [156], led to a significant reduction of cross-presentation and antigen translocation into the cytosol, indicating that sec61 is involved in this export process [78]. However, direct evidences are still missing and the question, which export channel is responsible for cross-presentation is controversially discussed.

2 Aim of this study

Cross-presentation is the possibility for DCs to present extracellular antigens on MHC-I molecules. One of the major pathways for this presentation is the "endosome to cytosol" pathway. During this, the antigens are internalized and have to be translocated into the cytosol for proteasomal degradation.
One of the main unanswered questions of this cross-presentation pathway is, how the antigens can overcome the membrane barrier between the endosomal compartment and the cytosol. Therefore, the machinery regulating such an non-canonical protein transport across endosomal membranes is intensively and controversially discussed.

The aim of this study was to clarify, whether the ERAD machinery is involved in cross-presentation and which channel protein enables the extracellular antigen to pass the membrane barrier into the cytosol for proteasomal degradation and further processing for MHC-I presentation. Especially the role of sec61 in context of cross-presentation was supposed to be analyzed in greater detail. This channel protein has already been reported to be involved in the ERAD system, transporting proteins in the same direction as needed for antigen export [151, 152]. Furthermore, it was in the center of interest, whether sec61 or other components of the ERAD machinery can be recruited to the antigen-containing compartments. For these analyzes we used a novel method of endosomal flow cytometry [157] in combination with ER-retaining antibodies (intrabodies).

As it is known that recruitment of other ER-components to the site of cross-presentation is strictly controlled and TLRs have an influence on cross-presentation in general [106, 158], we additionally planed to investigate the role of TLR-signaling during the antigen translocation into the cytosol.

3 Material and Methods

3.1 Material

Here, the used material and equipment is listed. Additionally, the procedures during the different experiments are described below.

3.1.1 Technical equipment

Device	Specification and company
Flow cytometry	LSRII, BD Bioscience
Cell-homogenisator	Wheaton Dure-Grind steal-dounce
NanoDrop2000 spectrophotometer	Thermo Scientific
ELISA plate reader	Molecular Devices
Electroporator	BioRad GenPulser X-cell, Biorad
Microscope	ApoTome, Carl Zeiss
ChemiDoc MP imaging system	BioRad
Centrifuge 5417R	Eppendorf
Ultracentrifuge	Beckman

3.1.2 Consumables

All not listed consumables were purchased from Sarstedt and Eppendorf.

Consumable	Company
6-well plates	TPP
24-well plates	TPP
Non-treated 24-well plates	Costar
96-well plates	TPP
ELISA plates, Microlon 96-well flat bottom	Greiner
1 l BottleTop vacuum filter (0.45 µm pore)	Corning
Sterile syringe filter, 0.20 µm and 0.45 µm, CA	Whatman
Falcon cell strainer, 0.40 µm	BD Bioscience
Filter pipette tips	Axygen

Consumable	Company
Cyrotubes	Nunc
Electroporation cuvettes	BioRad
PD10 desalting columns	GE Healthcare
Zebra desalt spin columns	ThermoFisher
Nitrocellulose membrane	Whatman
Filter paper	Whatman
Ultracentrifugation tubes	Beckman

3.1.3 Chemicals, reagents and experimental kits

All chemicals and reagents, as far as not otherwise marked, were purchased from Sigma-Aldrich in analytic quality.

General reagents

Chemicals	Company
Sulfo-NHS-LC-biotin	ThermoFisher
Complete EDTA-free protease inhibitor	Roche
Neutravidin-horseradish peroxidase	ThermoFisher
IL-2, recombinant ELISA-standard	eBioscience
IGEPAL	Sigma-Aldrich
Low-fat dry milk powder	Applichem
Tween-20	Sigma-Aldrich
Coomassie staining	Roth
Bovine serum albumin (BSA)	Roth
Bromphenol blue	Roth
AnnexinV buffer	BD Bioscience
Fluromount	Sigma-Aldrich

Molecular biology

Reagents	Company
Protein A/G PLUS-agarose	Santa Cruz
DynBeads ProteinG	Invitrogen
SYBR Safe DNA gel stain	Invitrogen
AccuPrime DNA-polymerase	Invitrogen
Phusion High Fidelity DNA-polymerase	New England Biolabs GmbH
Superscript II (reverse transcriptase)	Invitrogen

Reagents	Company
RNeasy Mini Kit	Qiagen
ProteoExtract®SubcellularProteome Extraction kit	Calbiochem
mMESSAGE mMACHINE T3 kit	Ambion, Applied Biosystems
mMESSAGE mMACHINE T7 ultra kit	Ambion, Applied Biosystems
Poly-A tailing kit	Ambion, Applied Biosystems
DNA-ladder 1 kb	New England Biolabs GmbH
Phosphate buffered saline (PBS)	Biochrom
Ampicillin	Carl Roth
Kanamycin	Carl Roth
Restriction enzymes	New England Biolabs GmbH
NucleoBond Xtra Midi kit	Macherey-Nagel
NucleoSpin Xtract kit	Macherey-Nagel
Protein ladder	New England Biolabs GmbH

Cell biology

Reagents	Company
Lipofectamine2000	Invitrogen
MHC-I OVA peptide (257-264)	Tebu-Bio
MHC-II OVA peptide (323-339)	Tebu-Bio
IC fixation buffer	eBioscience
Permeabilization buffer 10x	eBioscience
Fixation/Permeabilization concentrate (4x)	eBioscience
DAPI for nucleic acid staining	Sigma-Aldrich
Hoechst 33258	Invitrogen
Ovalbumin (OVA)	Serva
Endotoxin-free (endograde) OVA	Hyglos
OVA-alexa 488, 568 and 647	Invitrogen
MG132 (z-Leu-Leu-Leu-Al)	Sigma-Aldrich
Transferrin-alexa 647	Invitrogen
β-lactamase (Enterococcus cloacae)	Sigma-Aldrich
CCF4 FRET substrate	Life technologies
Cytochrome c (horse)	Sigma-Aldrich
Polybrene (Hexadimethrine bromide)	Sigma-Aldrich
Eeyarestatin I	Santa Cruz
ExotoxinA	Sigma-Aldrich
BrefeldinA	Sigma-Aldrich

Cell culture

Reagents	Company
OptiMEM	Invitrogen
DMEM, high glucose (4,5 g/l), with L-glutamine	PAA
IMDM, with L-glutamine	PAA
RPMI-1640, w/o L-glutamine, with HEPES	PAA
Sodium pyruvate, 100 mM	PAN
L-glutamine, 200 mM	PAN
NEAA (100 x)	PAN
β-mercaptoethanol	Sigma-Aldrich
Trypsin 0,05 % / EDTA 0,02 %	PAN
FCS Clone	PAA
Penicillin/Streptomycin	PAA
EDTA 2 mM	Sigma-Aldrich

3.1.4 Antibodies

Primary Antibodies

Antigen	Clone / Lot	Company	Usage	Dilutions
IL-2 purified	JES6-1A12	eBioscience	ELISA	1:1000
IL-2 biotinylated	JES6-5H4	eBioscience	ELISA	1:1000
Rab5	D-11	Santa Cruz	IF, FlowCyt	IF 1:200, FlowCyt 1:100
Sec61α	07-204	Upstate	WB	1:1000
Sec61β	ab78276	Abcam	IF	1:200
Sec61γ	11147-2-AP	Proteintech	WB	1:500
Derlin1	derlin1-1	Sigma-Aldrich	WB, FlowCyt	WB 1:1000, FlowCyt 1:200
EEA1	H-300	Santa Cruz	IF	1:200
Lamp	1D4B	eBioscience	IF	IF 1:500, FlowCyt 1:1000
GM130	—	Sigma-Aldrich	IF	1:250
AnnexinV	—	BD Bioscience	FlowCyt	5µl/stain
Actin	AA20-33	Sigma-Aldrich	WB	1:5000
Calnexin	ab22595	Abcam	WB	1:1000

Antigen	Clone / Lot	Company	Usage	Dilutions
IgG isotype	P3.6.2.8.1	eBioscience	FlowCyt	depending on primary antibody concentration
HIS-tag	MAC1396	Serotec	WB	1:1000
Myc-tag	9E10	Biolegend	WB	1:1000
H-2Kb-SIINFEKL	25.D1.16	eBioscience	FlowCyt	1:60

Secondary antibodies

Antibody	Company
α-mouse-IgG-alexa 568	Invitrogen
α-rabbit-IgG-alexa 488	Invitrogen
α-rabbit-IgG-alexa 568	Invitrogen
α-rabbit-IgG-alexa 647	Invitrogen
α-mouse-IgG-HRP	Santa Cruz
α-rabbit-IgG-HRP	Santa Cruz
α-human-IgG-HRP	Santa Cruz

All alexa-coupled antibodies were used in a 1:1000 dilution, all Santa Cruz antibodies were diluted 1:5000.

3.1.5 Primers and cloning vectors

Primers

All primers were purchased from Life technologies.

cDNA production

Name	Sequence
oligo-dt-T7	TTTTTTTTTTTTTTTT

mRNA production

Name	Sequence
EGFP forw.	TAGTGAACCGTCAGATCCG
EGFP rev.	TAATACGACTCACTATAGGG

RT-PCR

Name	Sequence
GAPDH forw.	CAGCAATGCATCCTGCAC
GAPDH rev.	GGATGACCTTGCCCACAG
Sec61α1 forw.	CCGAGTGGACCTTCCAATC
Sec61α1 rev.	CACTGAGCCGAACGATTCC
Sec61α2 forw.	TCGTGTTGACTTGCCCATT
Sec61α2 rev.	AAATATGGCTCCCATAGACTCAG

Cloning strategies

Name	Sequence
Cloning into the target vector pEGFP-C3	
Sec61β forw.	GATCAAGTTCATGCCGGGTCCAACGCCCAGTG
Sec61β rev.	GATCGTCGACCATGATCGCGTGTACTTGCCCC
Cloning into the target vector pCMV-tag2B	
IB forw.*	GCGCCGCGGGAGCTCGCCACCATGGGATGGAGCTGTATCATCCTCTTC TTGGTAGCAACAGCTACAGGCGCGCACTCCCAGGTGCAGCTG
IB bicist rev.	GCGCGAATTCGTCGACTCAGGAGAGCACACACTTGCAGCTC
IB mono with KDEL rev.	GCGCGAATTCGTCGACTTATAGTTCGTCCTTATGATGATGG
IB mono w/o KDEL rev.	GCGCGAATTCGTCGACTTAATGATGATGGTGATGATGGGATAG
CD3δ -SIINFEKL forw.	GCCGGCGCGGCCGCAAGCTTGCCACCATGGAACAC AGCGGGATTCTGGCTAGTC
CD3δ-SIINFEKL rev.	GAGGGAACTGGCCCCGGAACAAGAAATCTGCCCTTGAGCAGCTTGAGAG TATAATCAACTTTGAAAAACTGACTGAATGGACCAGTTAACTCGAGGGCCG
TCRα -SIINFEKL forw.	GCCGGCGCGGCCGCAAGCTTGCCACCATGGACAA GATCCTGACAGCATCGTTTTTAC
TCRα-SIINFEKL rev.	GAGGCTCCAGCAAAGCCCTGCTGTGGGCGCCCTTGAGCAGCTTGAGAGTA TAATCAACTTTGAAAAACTGACTGAATGGACCAGTTAACTCGAGGGCCGG
Cloning into the target vector pCMV-LV	
Sec61β-GFP	sec61β-GFP was cloned from pEGFP-C3 into pCMV-LV by AgeI and SalI
IB bicist forw.	GCGCCCGCGGGGCGCGCCGCCACCATGGGATGGAGCTGTATCATCCTC
IB bicist rev.	GCGCGAATTCCTCGAGTCAGGAGAGCACACACTTGCAGCTC

Name	Sequence
Cloning into the target vector pEGFP-C3	
IB mono forw.	GCGCCGCGCGCGCCGCGGGCCACCATGGGATGGAGCTGTATCATCCTC
IB mono rev. **	GCGCGAATTCCTCGAGGGCAACTAGAAGGCACAGTCGAGGC
IB mono rev. ***	GCGCGAATTCCTCGAGGGTACCGGGCCCCCCCTCGAGGT

* This primer was used for all intrabody (IB) constructs (AMA56 as well as all SH814-A2 variants). Alternatively to the cloning of mono-IB constructs into the lentiviral vector also an elimination of the GFP sequence in the pCMV-LV-bicist-IB by NheI digest was performed.

** Used for cloning with pKD-bicist-SH814-A2 or pKNC-mono-SH814-A2 vector as template.

*** Used for cloning with IB-constructs within a pCMV-tag2B vector as template.

Sequencing primers

For DNA-sequencing the standard primers of GATC were used.

Vectors

Name	Resistance	Origin
pEGFP-C3	Kanamycin	Clontech
pCMV-tag 2B	Kanamycin	Clontech
pCMV-LV	Ampicillin	Addgene
pCMV-gag	Ampicillin	Addgene
pCMV-VSVG	Ampicillin	Addgene
pCMV-GFP	Kanamycin	AG Burgdorf
pCMV-OVA-flag	Kanamycin	AG Burgdorf
pKD-mono-AMA56 (control vector)*	Ampicillin	University of Braunschweig
pKD-bicist-AMA56 (control vector)*	Ampicillin	University of Braunschweig
pKD-bicist-SH814-A2 (with KDEL)*	Ampicillin	University of Braunschweig
pKD-mono-SH814-A2 (with KDEL)*	Ampicillin	University of Braunschweig
pKNC-bicist-SH814-A2 (wo KDEL)*	Ampicillin	University of Braunschweig
pKNC-mono-SH814-A2 (wo KDEL)*	Ampicillin	University of Braunschweig

* The "bicist" vectors contain an IRIS-GFP sequence, which is missing in "mono" vectors.

3.1.6 Buffers and solutions

If not otherwise noted, the pH of the solution was adjusted by HCl or NaOH and the solutions were stored at RT.

Substrates for bacteria culture

TFB I
NaAc, pH = 6, 30 mM
$MnCl_2 \circ 4\ H_2O$, 50 mM
NaCl 100 mM
$CaCl_2$ 10 mM
glycerol 15 % (v/v) pH = 6, sterile filtered

TFB II
MOPS, pH = 7, 10 mM
NaCl 10 mM
$CaCl_2$ 75 mM
glycerol 15 % (v/v) pH = 7, sterile filtered

YB-media 2x
YT-Media 98 %
KCl (Stock 1 M) 10 mM
$MgSO_4$ (Stock 2 M) 20 mM
20 min at 121 °C autoclaved
stored at 4 °C

YT-media (2x)
NaCl 0,5 %
Bacto Yeast Extract 1 %
Tryptone 1.6 %
pH = 7.5; 20 min at 121 °C autoclaved
stored at 4 °C

LB-media
Tryptone 10 g/l
Yeast extract 5 g/l
NaCl 5 g/l
20 min at 121 °C autoclaved
stored at 4 °C

Ampicilin was used with a final concentration of 100 μM/ml, kanamycin with 50 μM/ml.

DNA-preparation and analysis

Mini-prep. solution 1
Tris/HCl, pH = 8, 50 mM
EDTA 10 mM
RNaseA 100 μg/ml
stored at 4 °C

6x DNA-loading buffer
Bromphenol blue 0,25 %
Xylencyanol blue 0,25 %
Glycerol 30 %
EDTA 50 mM

Mini-prep. solution 2
NaOH 200 mM
SDS 1 %

Mini-prep. solution 3
KAc 3 M (pH=5.5)

TAE-buffer (50x)
Tris 2 M
acetic acid 1 M
EDTA 0,05 M
pH = 8,3

DNA-transfection

2x BBS buffer
BES 50 mM
NaCl 280 mM
Na_2HPO_4 1.5 mM
pH = 7
stored at -20 °C

$CaCl_2$
$CaCl_2$ 2.5 M (highest purity)
stored at 4 °C

Transfections in 6-well or 24-well plates were done by Lipofectamine2000 according to the standard lipofectamine transfection protocol.

Protein analysis

Phosphate-lysis-buffer (pH=8)
$NaPO_4$ 20 mM
NaCl 140 mM
$MgCl_2$ 3 mM
NP-40 0.5 %
Complete protease inhibitor freshly added
stored at 4 °C

TEA-lysis-buffer
NaCl 150 mM
$MgCl_2$ 1 mM
$CaCl_2$ 1 mM
TEA 10 mM
TritonX 1 %
Complete protease inhibitor freshly added

4x Laemmli buffer
Tris-HCl (pH = 6,8) 250 mM
SDS 8 %
Glycerol 40 %
Bromphenol blue 0.08 %
(10 % β-mercaptoethanol)
stored at -20 °C

SDS-PA-stacking gel
dH_2O 1.2 ml
Rotiphorese-Mix 330 μl
Tris-HCl (Stock 1 M, pH = 6.8) 250 μl
SDS (10%) 20 μl
APS (10%) 20 μl
TEMED 2 μl

SDS-PA-resolving gel 10 %
dH_2O 1.9 ml
Rotiphorese-Mix 1.7 ml
Tris-HCl (Stock 1.5 M, pH = 8.8) 1,3 ml
SDS (10 %) 50 µl
APS (10 %) 50 µl
TEMED 2 µl

Towbin-buffer (10x)
Glycine 1.92 M
Tris 250 mM
pH = 8.53
(if needed 20 % methanol was added)

SDS-running buffer (10x)
Glycine 1.92 M
Tris 250 mM
SDS 1 %

Coomassie staining solution
MeOH 40 %
$C_2H_4O_2$ 10 %
Coomassie 0,1 %

TBS buffer
Tris-HCl (Stock 1 M, pH = 7.5) 50 mM
NaCl 150 mM

Wash buffer (TBS-Tween)
Tris-HCl (Stock 1 M, pH = 7.5) 50 mM
NaCl 150 mM
Tween20 0.05 %

ELISA-buffer

ELISA coating buffer
$NaHCO_3$ 0,1 M, pH = 8.2

ELISA blocking buffer
BSA 1 % in PBS

ELISA washing buffer
Tween20 0.05 % in PBS

ABTS buffer
Citric acid 0.1 M, pH = 4.35

ABTS development solution
ABTS buffer including 1 mg/ml ABTS and
15 µl H_2O_2 (30 %) per 10 ml

Endosome preparation and β-lactamase buffers

Cell homogenization/endosomal flow cytometry buffer
MES 25 mM
NaCl 150 mM
pH = 6.5
Complete protease inhibitor was freshly added, sterile filtered

β−Lactamase EM-buffer
KCl 7 mM
NaCl 120 mM
$CaCl_2$ 1.8 mM
$MgCl_2$ 0.8 mM
Glucose 5 mM
HEPES 25 mM, pH = 7.3
1 mM protease inhibitor Probenicid was freshly added

β−Lactamase CCF4 loading buffer
Sol.A 12 µl (contains 1 µM CCF4) *stored at -20 °C*
Sol.B 60 µl
mixed well
Sol.C 928 µl
mixed well
Sol.D 50 µl
β-Lactamase EM-buffer with Probenicid 4950 µl

3.1.7 Bacteria strains, cell lines and mice strains

Bacteria	Strain	Genotype
Escherichia coli	DH5α	F-, φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1
Escherichia coli	XL-1 blue	endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)

Cell line	Description	Culture conditions
HEK293T	Human embryonic kidney cells infected with adenovirus E1A/B	DMEM (4.5 g/l glucose), 10 % FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 1 mM sodium pyruvate. Cells were harvested by Trypsin/EDTA in PBS.
DC2.4	Murine, immortalized and immature dendritic cells [159]	RPMI 1640, 10 % FCS, 50 µM β-mercaptoethanol, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate and 1 % non essential amino acids. Cell harvesting was done by 2 mM EDTA in PBS.

Cell line	Description	Culture conditions
J558L-GMCF	Transgenic BALB/c myeloma cell line producing and secreting GM-CSF into the supernatant.	RPMI 1640, 10 % FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin. These cells contain a G418 resistance for selection and are subcultured by dilution into fresh media.

Primary cells	Description	Culture conditions
BMDCs	Bone marrow-derived dendritic cells, differentiated from the bone marrow of the relevant mouse strain	IMDM, 10 % FCS, 2.5 % GM-CSF-containing supernatant of J558L cells, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The culture was done as described below.
T-cells	OT-I or OT-II T-cells gained out of the spleen of the correspondent mice	RPMI 1640, 10 % FCS, 50 μM β-mercaptoethanol, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate.

Mouse strain	Description
C57BL/6	Wild-type strain
TRIF -/-	Contains a point-mutation in the gene encoding for TRIF-/-, which leeds to a frame shift and a non-functional, truncated TLR-TRIF signaling molecule. The TRIF-/- mice have a C57BL/6 genetic background.
MyD88 -/-	Is missing the TLR-MyD88 signaling molecule. The mice have a C57BL/6 genetic background.
OT-I RAG1 -/-	Only produces $CD8^+$ T-cells, recognizing the OVA-peptide (257-264) on H-2Kb MHC-I molecules.
OT-II	Produces $CD4^+$ T-cell recognizing the OVA-peptide (323-339) on I-Ab MHC-II molecules.

For all experiments 8 to 16 weeks old mice were used. These were bred under specific pathogen-free conditions. The treatment of the animals was in accordance with local animal experimentation guidelines.

3.1.8 Software

Software	**Company**
Papers 2	Mekentosj
FlowJo V8.7	TreeStarInc
FACS-DIVA	BD Biosciences
ImageJ	National Institute of Health
Axiovision	Zeiss Software
Photoshop CS6	Adobe Systems
Illustrator CS6	Adobe Systems
Microsoft Office Mac 2011	Microsoft
Image Lab Software	Bio Rad
Prism 6	GraphPad Software
Lyx	open source
Serial Cloner 2-5	SerialBasics

3.2 Methods

3.2.1 Molecular-biological methods

3.2.1.1 Generation of transformation competent bacteria

For the production of chemical competent bacteria the method of Hanahan *et al.* was used [160]. Therefore, 3 ml of bacterial pre-culture were grown overnight at 37 °C in LB medium. Subsequently, this culture was extended to 100 ml in YB medium and stopped dividing during their exponential growth period (optical density of 0.45 - 0.55 OD600) by cooling on ice for 10 min. The bacteria were centrifuged for 10 min with 3220 rcf and the pellet was resuspended in 20 ml TFB-I. After 10 min of incubation on ice, the bacteria were spun down again (3220 rcf, 10 min) and finally taken up in 4 ml TFB-II buffer. Aliquots of 100 μl were shock frozen with liquid nitrogen and stored at -80 °C.

3.2.1.2 RNA-extraction and cDNA synthesis

The RNA isolation out of the relevant cells was done as described in the Qiagen RNeasy Mini-Kit protocol. Subsequently, the reverse transcription of mRNA was prepared and incubated at 42 °C for 90 min. The storage of synthesized cDNA was done at -20 °C.

Reaction mixture		Stock-solution
RNA	5 μl	
RNase free H_2O	5.5 μl	
RNAsin	0.5 μl	
5x reaction buffer	4 μl	
0.1 DTT	2 μl	5 mM
10 mM dNTPs	1 μl	each 0.5 mM
Oligo dT primer	1 μl	25 μg/ml
Superscript II	1 μl	10 units/ml

3.2.1.3 DNA amplification (PCR) and analysis

Polymerase chain reaction The amplification of DNA was done by polymerase chain reaction. Therefore, prove-reading polymerases were used to avoid mutations inside of the amplificates. The reaction conditions were chosen as indicated by the instructions of the used polymerase. The annealing temperature was specifically adapted to the used primer constructs. The PCRs for cloning strategies contained 30 cycles of amplification, while PCRs, which were done to determine relative expression levels of specific gens, run only for 25 cycles.

Reaction mixture		
H_2O	36.5 μl	—
5x Reaction buffer	10 μl	—
Primer	1 μl	each 0.5 μM
Template	1 μl	0.5-1 μg cDNA 50-100 ng DNA-Plasmid
dNTP	1 μl	each 0.2 mM
Phusion polymerase	0.5 μl	0.02 units/μl

DNA analysis and purification by agarose gel electrophoresis Gel electrophoresis was done as described by Sambrook *et al.*. Therefore, the negative charge of DNA was used to separate DNA fragments by size. The agarose gels contained 0.8 % to 2 % agarose in 1x TAE buffer, depending on the expected DNA length. Additionally, SyBR-Safe DNA gel stain for the detection of the DNA bands by UV light was used. The size of DNA fragments was determined by 100 bp or 1 kb markers. For further processing of the DNA, the gel, containing the DNA of interest, was sliced out and purified regarding the instructions of the NucleoSpin Extract KitII. The elution was done with pure H_2O.

3.2.1.4 Cloning protocols and plasmid generation

Restriction digest For the generation of plasmid constructs, the DNA insert and the targeted plasmid were cut by the same or by matching restriction enzymes. For the DNA inserts, the restriction sides were added by PCR with primers containing the required cleavage sides. Additionally, the DNA was purified by Nucleo Spin Extract KitII before the sample was used for further processing. The reaction-mixture was incubated for 1 hr at 37 °C, if not otherwise indicated in the restriction enzyme descriptions. If the usage of both enzymes at once was not possible, the incubations were done sequential. As soon as the cutting of the DNA was completed, it was again purified by agarose gel electrophoresis and Nucleo Spin Extract KitII.

Reaction mixture for restriction digest

PCR product or 1 μg plasmid in H_2O	28 μl
Appropriate 10x reaction buffer	4 μl
10x BSA	4 μl
Enzyme 1	2 μl
Enzyme 2	2 μl

Ligation For the ligation a 5' phosphate on the one side and a 3' OH on the other side were required to connect the two DNA fragments. The fusion of this two groups was done with the T4 DNA-ligase by ATP hydrolysis. Therefore, the reaction mixture was incubated for at least 2 hrs at room temperature or overnight at 16 °C.

Reaction mixture for ligation

PCR fragment	5.5 μl
Vector	3 μl
T4 DNA-ligase buffer	1 μl
T4 DNA-ligase	0.5 μl

Transformation of chemical competent bacteria For transformation, 50 μl of competent bacteria, which were thawed on ice before, were added to the whole ligation of newly cloned constructs or 10 ng of already purified plasmid. After 30 min of incubation on ice, the bacteria were heated to 42 °C for 90 sec and cooled for one additional minute on ice. Sequentially, 1 ml antibiotic-free LB-medium was added and the incubation was done for 1 hr at 37 °C on a shaker. After short spinning, the pellet was resuspended in a small amount of LB media and the bacteria were plated on LB agar plates, containing the correspondent antibiotic. For the growth of colonies the incubation was done overnight at 37 °C.

Analytical DNA preparation This preparation was used to identify positive colonies on LB agar plates. Selected colonies were picked from the LB agar plate and transferred into a 15 ml falcon, containing 3 ml LB media with the corresponding antibiotic. After incubation overnight at 37 °C, the bacteria suspension was transferred and pelleted in an Eppendorf reaction tube (21000 rcf, 5 min). Subsequently, the pellet was resuspended in 100 μl Mini-preparation buffer I (resuspension buffer) and mixed with 200 μl Mini-preparation buffer II (an alkalic lysis buffer) by inverting the tube three times. After 5 min of incubation, the Mini-preparation buffer III was added for neutralization of the alkaline lysis buffer and the sample was again mixed by inverting the tube. Thereafter, the lysate was spun down for 5 min at 21000 rcf to separate the plasmid DNA from bacteria fragments and genomic DNA. The supernatant was transferred into a new tube and mixed well with 1 ml ice cold 100 % pure ethanol as well as 50 μl Mini-preparation buffer II by vortexing. The precipitated DNA was pelleted for 30 min with 21000 rcf at 4 °C. After washing one time with 70 % ethanol, the DNA was centrifuged again (21000 rcf, 5 min) and the pellet was dried at 37 °C. Resuspension was done in 25 μl pure H_2O. The gained plasmid DNA was tested for successful cloning by digesting with different restriction enzymes and gel electrophoreses. The enzyme combination was selected to achieve a definitive identification of a successful insert integration.

Preparative DNA purification The bacteria with successful cloned plasmids were expended overnight in 300-500 ml LB medium and pelleted in 50 ml falcons by centrifugation. The preparation was done as described in the instructions of the NucleoBond Xtra Midi Kit. Afterwards, the plasmid-pellet was resuspended with pure H_2O and the concentration was measured with the Nanodrop2000 at 260 nm. The DNA purity was determined by the ratio of the absorption at 260 nm and 280 nm, aiming a ratio of 1.7 to 1.9. The sequences of the cloned constructs were analyzed by GATC Biotech AG.

3.2.1.5 *In-vitro* mRNA production out of DNA templates

For mRNA production a PCR was done on the gene encoding plasmid (for GFP) or the plasmid was spliced by restriction enzyme digest (BamHI digest on the OVA encoding plasmid). Important for the template DNA was that a T3 or a T7 binding side was present on it, where the transcription enzyme can bind. After purification by Nucleo Spin Extract KitII, mRNA synthesis was done as described in the instructions of the T3 or T7 mMESSAGE mMACHINE kit. Additionally, a polyA tail was added after successful mRNA synthesis by a poly-A tailing kit according to the kit protocol. For final purification of the mRNA, the RNeasy MiniKit of Qiagen was used. The synthesized mRNA was directly electroporated into cells for experimental analysis or stored in aliquots at -80 °C.

3.2.2 Biochemical methods

3.2.2.1 Cell lysis and western blot

Cell lysis For western blot analyses, the cells containing the proteins of interest were harvested, pelleted with 300 rcf for 5 min and washed with PBS. After repeated pelletizing and removement of the supernatant, the cells were lysed in cell-lysis buffer by detergents. The amount of cells as well as of the lysis buffer variated between the experiments. For quantitative protein expression analysis, $1.25 \circ 10^6$ cells per 100 μl lysis buffer were dissolved with PLP buffer. For immunoprecipitation $2.5 \circ 10^6$ cells per 100 μl TEA-lysis buffer were used. The lysate was incubated for 20 min on ice and afterwards spun down with 21000 rcf at 4 °C for 5 min to remove the remaining cell fragments. After transferring the supernatant into a new tube, 4x Laemmli buffer was added. If needed, β-mercaptoethanol was intermixed to reduce disulfide bonds within the protein of interest. The mixed samples were heated for 5 min at 95 °C. Subsequently, the samples were directly used or stored at -20 °C.

SDS gel electrophoresis For analysis of the lysate the method of SDS-page was used as described by Laemmli *et al.* [161]. Therefore, the protein structure was denatured and charges of the proteins were covered by SDS. This enabled a partition only regarding the size of the proteins. The separation was done in a polyacrylamide gel surrounded by SDS-containing running buffer. After concentration and collection of the samples within the stacking gel on the top of the separation gel by a low amperage of 15 mA per gel, protein separation was done by 30 mA per gel.

Western blot After gel separation, the proteins were transferred to a nitrocellulose membrane by western blotting. Therefore, the gel in combination with a nitrocellulose membrane was placed into a wet blotting chamber containing cooled Towbin blot buffer. For transfer constant 300mA were used for 1 hr. Subsequently, the membrane was blocked with 5 % milk powder in TBST buffer for 1 hr to avoid unspecific binding of other proteins or antibodies. After a short washing with TBST the antibody-staining was done. For this, TBST, containing the antibody in corresponding concentrations, in combination with 0.05 % milk powder was used. The incubation took place for at least 1 hr at RT or at 4 °C overnight. For neutravidin-Hrp staining no milk powder was added during the staining procedure. Thereafter, the membrane was washed three times and secondary antibody staining was done for 1 hr at RT. Finally, after threefold washing for 15 min with TBST, the membrane was incubated for 1-2 min with ECL substrate. This was processed by Hrp, which is coupled to the secondary antibody. The detection was done by x-ray film or by a ChemiDoc detection system.

Coomassie staining For the detection of all proteins inside of the lysate, Coomassie staining was used. Therefore, the SDS gel was incubated for at least 4 hrs in Coomassie reaction solution, which non-specifically marks all proteins by interaction with the basic side chains of the amino acids. The incubation was done at RT. After staining, the polyacrylamid gel was washed several times with H_2O to remove unbound Coomassie molecules. This washing was continued until clear bands were visible.

3.2.2.2 Immunoprecipitation

For immunoprecipitation of sec61α with the sec61α-binding intrabody construct, BMDCs were lysed with TAE lysis buffer. Additionally, they were homogenized during lysis by pulling the cells five times through a 27G needle. In parallel to this, anti-human IgG Dynabeads were loaded with 40 μg sec61α-binding intrabody construct, which was fused to a human IgG-Fc part. The loading took place at RT for 20 min. After cell lysis and removement of the remaining cell fragments with centrifugation (21000 rcf, 5 min), the lysate was distributed equally to the construct-loaded and washed beads or to control beads. This suspension was incubated at 4 °C under rotation overnight. Subsequently, the beads were separated from the lysate by magnetic force and the supernatant was carefully removed. After three times of washing, beads were transferred into a new tube and washed one more time. Next, 60 μl TAE lysis buffer mixed with β-mercaptoethanol and Laemmli buffer, were added to the beads. After 10 min elution by 70 °C, the beads were removed and the samples heated at 95 °C. The analysis were done by Coomassie staining and western blotting.

3.2.3 Cell-culture conditions

All cells were cultured at 37 °C with 5 % CO_2 by a humidity of 90 %. If not otherwise indicated, all incubation steps of cell culture experiments were done under this conditions. HEK293T cells as well as DC2.4 cells were cultured up to 60-70 % confluence before splitting was done for further culturing. HEK293T cells were harvested with a 0.05 % Trypsin / 0.02% EDTA combination, while DC2.4 cells were spit with 2mM EDTA only. Both cell lines were plated on cell culture dishes. BMDCs were cultured as described below and T-lymphocytes were directly used for experiments after isolation. All cells were spun with 300 rcf for 5 min. The complete work inside of the cell culture was done under sterile conditions. Additionally, the cell culture was tested mycoplasma-free within frequent time periods by the kit “Micotrace” (GE Healthcare).

3.2.3.1 Production of GM-CSF supernatant

The GM-CSF-containing medium for BMDC differentiation was produced by the transgenic J588L cell line, which secrets GM-CSF into its supernatant. These cells were freshly thawed and expanded for each production. Thereafter, $1 \circ 10^6$ cells were plated per 15 cm dish in 30 ml of their recommended medium and incubated for 8 to 10 days. After this incubation, the cells were spun down and the supernatant was filtered by a BottleTop-filter. The resulting sterile solution was aliquoted and stored at -20 °C.

3.2.3.2 Generation of BMDCs

For the generation of BMDCs, the bone marrow of a mouse was isolated by flushing it out of the hind limbs with PBS by using a 26G needle. The extracted cells were resuspended and filtered through a 40 μm cell strainer. After centrifugation, the cells were taken up in BMDC culture medium and plated on three 10 cm petri dishes per mouse. For each plate 15 ml culture medium were used. After three days of incubation, the cells were harvested. Therefore, the supernatant was collected and adherent cells were incubated for 5 min with 2 mM EDTA at 37 °C and detached by pipetting. Following pelleting and uptake in fresh culture medium, the cells were plated on six 10 cm petri dishes per mouse. BMDCs between culture-day 7 or 8 were used for all experiments, if not otherwise indicated.

3.2.4 Cell-biological methods

3.2.4.1 Production of lentivirus and transduction of DCs

Transfection of HEK293T cells For the generation of lentiviruses a three plasmid system was used. Therefore, $3 \circ 10^6$ HEK293T cells were plated on a 10 cm cell culture dish one evening before transfection was done and incubated overnight to get a 70 % covered dish. At the next morning, the supernatant was replaced by 5 ml fresh culture medium and the cells were incubated for additional 2 hrs. For transfection, a mixture of three plasmids was generated. Therefore, 10 μg of the transfer plasmid were used. This contained the gene construct, which was supposed to be included inside of the virus between the long terminal repeats (LTRs). Additionally, 10 μg packaging plasmid (pCMV-gag) encoding the viral genes gag (capsid protein), pol (reverse transcriptase as well as integrase) and RRE (rev-response element) were added. For the viral envelop 2 μg plasmid encoding the G-glycoprotein of the vascular stomatitis virus (pCMV-VSVG) were intermixed. These plasmids were subsequently transfected into HEK293T cells by a calcium-phosphate

precipitation. Therefore, the DNA was filled up to 450 μl with sterile H_2O and mixed well with 50 μl $CaCl_2$. The precipitation was done by adding the $CaCl_2$-DNA solution to 500 μl 2x BBS buffer and mixing it well by vortexing. After 1 - 10 min crystals formed and became visible under the microscope. These crystals were distributed drop-wise over the whole area of the cell culture dish.

Harvest and concentration of the lentivirus After incubation and crystal uptake overnight, the supernatant was carefully replaced by 12 ml fresh media. The virus producing cells were incubated for 24 hrs and the supernatant containing viral particles was harvested afterwards. This procedure was repeated once to gain the maximal amount of virus from one plate. The collected supernatant was spun down with 500 rcf and filtered through a 0.45 µl syringe filter to get rid of the cellular fragments. If needed, the volume of the viral solution was reduced and the virus concentrated by ultra-centrifugation. Here we used a SW-32Ti rotor with 21000 rpm (13000 rcf) for 2 1/2 hours. After centrifugation, the supernatant was discarded and the pellet resuspended in the culture medium of the cells, which were planned to be infected in the further experiment. The virus suspension can be stored for several days at 4 °C.

Transduction of DCs by lentivirus For transduction of BMDCs as well as DC2.4 cells the concentrated virus was used. Additionally to the needed additives of the culture medium, 10 μg/ml polybrene were added before the infection took place. For this, the cells were harvested and the needed number of cells (depending on the experiment) was pelleted with 300 rcf. The cells were resuspended in the virus-containing medium and cultured at 37 °C for 1 day. After this incubation time, the virus was removed and fresh culture medium was added.
For BMDCs, which are very resistant to viral infections, virus harvested from 3-6 HEK293T cell dishes was used per $1 \circ 10^6$ BMDCs. The infection was done during the splitting procedure at day three of their culture. Following to the infection, BMDCs were cultured for four additionally days and used at day 7 for all experiments. The DC2.4 cell-infection was done with the virus of one plate HEK293T cells per $1 \circ 10^6$ cells. After incubation overnight, the supernatant was replaced by fresh medium and the infected cells were cultured for at least three additional days before the experiments were performed.

3.2.4.2 OVA uptake assay

To control the antigen uptake of differently treated cells, they were incubated with 250 ng/ml fluorophore-labeled OVA for 15 min at 37 °C. After incubation, the cells were harvested with 2 mM EDTA and a cell scraper, washed once with PBS and analyzed by flow cytometry.

3.2.4.3 Enzyme linked immunosorbent assay (ELISA)

Cell culture for IL-2 production To analyze the efficiency of antigen presentation, differently treated DCs were plated in a 96-well plate with 50000 cells/well and incubated, if not otherwise notified, with the model antigen OVA for 2 hrs. After antigen uptake and processing, the supernatant was discarded and the cells were washed once with T-cell medium to remove the remaining antigens. In experiments, where the incubation time of the treated DCs needed to be limited to 2 hrs, the cells were fixed before the T-cells were added. For fixation, the DCs were washed three times with PBS and incubated for 60 sec with 0.08 % glutaraldehyde - PBS solution. After fixation, the cells were washed again three times with PBS and once with T-cell culture medium.
To detect the antigen presentation on DCs, OT-I or OT-II T-cells were added recognizing the MHC-I OVA-peptide complexes (by OT-I) or the MHC-II peptide presentation arrangements (by OT-II). These cells were gained out of the spleen of the corresponding mice. Therefore, the spleen was triturated by a steel cell-strainer and the fragments were well resuspended in PBS by pipetting. Subsequently, the cells were transferred into a 50 ml falcon through a 40 μm cell-strainer and the cell number was determined. After centrifugation of the T-cells, they were resuspended in the appropriate volume of T-cell medium. For incubation with the DCs, $0.1 \circ 10^6$ OT-I cells in 100 μl medium or $0.125 \circ 10^6$ OT-II cells in 100μl medium were added to each well. By detection of the peptide complexes via the T-cell receptor, the T-cells are activated and the IL-2 secretion is induced. After incubation overnight, the supernatant was harvested for the measurement of the IL-2 concentration. The IL-2 amount in the supernatant thereby allows conclusions about the T-cell activation, which again represents the antigen presentation efficiency of the DCs.

ELISA The determination of the IL-2 amount was done by a sandwich ELISA. All incubation steps took place at 37 °C for 1 hr or alternatively at 4 °C overnight, if not otherwise mentioned. Between all steps, the plate was washed three times with 150 μl washing buffer. For IL-2 detection the bottom of a special ELISA 96-well plate was covered with the coating buffer containing an IL-2 antibody. Per well 50 μl solution with 0.5 μg/ml antibody were used. Afterwards, the plate was washed and sequentially 150 μl blocking solution per well were incubated for 30 min at RT. The blocked plate was washed again and the collected T-cell supernatant was added into the coated wells (50 μl per well). To determine the exact concentrations of IL-2 within the samples, an IL-2 standard was placed on the plate. After incubation, the plate was washed and a second IL-2 antibody was added. Importantly, this antibody had a different binding-side against IL-2 as the first one. Additionally, it was coupled to a biotin molecule. The secondary antibody was used in the same concentration as the first one and the binding

occurred during standard incubation conditions. For detection, the plate was washed, 50 μl of a neutravidin-coupled Hrp (1mg/ml stock solution) per well were incubated for 30 min at RT and afterwards washed away again. The analyses were done by adding ABTS-reagent soluted in ABTS-buffer. This substance was processed by the peroxidase and was detectable at 405 nm. The read-out by the plate reader was done between 1 min and 15 min after adding ABTS, depending on the intensity of the signal.

3.2.4.4 DC electroporation

siRNA electroporation To knock down single proteins in DCs a siRNA (small interfering RNA) approach was used. For this, different siRNA molecules were generated matching only to the mRNA sequence of the targeted protein. After siRNA-binding, the mRNA was degraded and consequently the protein production inhibited.
To permeabilize BMDCs for siRNA-transfection, electroporation was used. Therefore, a determined electrical pulse was set on the solution, containing the DCs as well as siRNA. For transfection, the DCs were harvested between culture day 4 and 7 depending on the incubation time used for siRNA-mediated protein down-regulation. The electroporation time point was selected in a way that the experiments itself could be done at culture day 7 or 8. The harvested DCs were washed once with OptiMEM and the cell number was determined. For electroporation $4 \circ 10^6$ cells in 100 μl OptiMEM were used. Before cells were added, 10 μg in H_2O soluted siRNA were propounded in a 4 mm electroporation cuvette. The siRNA-cell mixture was incubated for 3 min and pulsed two times by 1000 V for 0.5 sec. Between the pulses was a time space of 5 sec. After electroporation, the cells were immediately transferred into pre-warmed culture medium and incubated until culture day 7 or 8 at 37 °C with 5 % CO_2 for upcoming analysis.

mRNA electroporation The synthesized mRNA of OVA or GFP was electroporated into the cells by a protocol similar to siRNA electroporation. The cells were harvested and washed with OptiMEM. For electroporation, the mRNA was added into a 4 mm electroporation cuvette and adjusted to a H_2O solution of 20 μl. The amount of the used mRNA depended on the experiment and is pointed out in the correspondent figure legends. The propound mRNA was mixed with 180 µl cell suspension in OptiMEM. For the endogenous presentation assay $0.2 \circ 10^6$ cells per electroporation were used. After mixture of the cells with mRNA, the electroporation was done immediately with 300 V for 6 msec. The mRNA-transfected DCs were afterwards directly used for different experiments.

3.2.4.5 Usage of different inhibitors

For inhibitor assays, the cells were pre-incubated with the respective inhibitor for at least 15 min before an experiment was started. During the experiment, the inhibitors were present during all incubation steps. For ELISA analysis, the cells were incubated with OVA in presence of the inhibitor, washed three times with PBS and fixed afterwards, to avoid the cell death of the DCs and an inhibitor contact to the T-cells. These were added for presentation analyses. ExotoxinA was used in a concentration of 10 μg/ml and Eeyarestatin I in a concentration of 3 μM. In the context of sec61-recruitment assays BrefeldinA was used with 10 μg/ml.

3.2.4.6 Analysis of ERAD function by the HEK-Venus cell line

The analysis of the function of the ERAD machinery was done by a cell line kindly provided by Prof. Cresswell [162]. This cell line was expressing a split version of the fluorescence protein Venus with one half localized in the ER and the other one in the cytosol. Importantly, the protein can only reunion and emit fluorescence light, when the ER-part has successfully been exported via the ERAD machinery. For analysis, differently treated HEK cells were incubated in presence of the proteasome inhibitor MG132 with a final concentration of 5 μM for 6 hrs to block proteasomal degradation and get functional Venus proteins in the cytosol. After incubation, the cells were harvested with 2 mM EDTA in PBS and washed once with pure PBS. Subsequently, analysis was done by flow cytometry.
For positive control, the HEK cells were treated with the inhibitor ExoA for 1 hr and subsequently incubated 6 hrs with MG132 in presence of the inhibitor, before flow cytometric analysis was done. Alternatively, the HEK cells were transfected with siRNA following the instructions of the lipofectamine2000 protocol. One day after transfection, the supernatant was exchanged and the cells were incubated for another day. Thereafter, MG132 was added for 6 hrs and the analysis was done as described above.

3.2.5 Antigen export assays

3.2.5.1 Isolation of cytosolic fractions to analyze the export of OVA

Biotinylation of OVA To analyze the antigen export of the model antigen OVA, it was biotinylated to enrich the exported cytosolic OVA and detect it by western blotting. Therefore, OVA was dissolved in PBS with a concentration of 100 mg/ml and spun down with 3220 rcf to remove unsolved OVA. Afterwards, OVA was purified by gel filtration (PD10 columns) to get rid of small OVA-fragments. Subsequently, the filtered OVA-solution was diluted to a concentration of 8 mg/ml. Of this solution 620 μl were mixed with 180 μl biotin reagent. For this biotin solution, 10 μg freshly opened Sulfo-NHS-LC-biotin were

solved in 180 μl H_2O. The mixture with OVA was incubated for 2 hrs at RT. Finally, the unbound biotin was removed by Zeba spin column filtration as described in the product instructions.

Isolation of exported OVA For OVA export 0.5 mg/ml biotinylated OVA were incubated for 45 min with differently treated DCs in presence of 5 μM MG132 to inhibit proteasomal degradation. After this incubation, the cells were harvested and collected in a 15 ml falcon. While 1/10 of the cells was removed for the total OVA-uptake control and lysed for western blot, 9/10 of the cells were pelleted and the cytosolic fraction was isolated by the subcellular proteome extraction kit of Calbiochem. Therefore, the cells were washed twice with washing buffer and afterwards lysed in 500 μl lysis buffer of the kit, containing 2.5 μl protease inhibitor. The lysate was incubated on ice for 1 min and spun down for 5 min with 500 rcf at 4 °C. The supernatant, containing the cytosolic fraction, was carefully transferred into a new tube and OVA-biotin in the cytosolic fraction was enriched by affinity chromatography using 50 μl PBS-washed neutravidin-agarose beads. The incubation was done for 1 hr at 4 °C under rotation. Afterwards, the agarose beads were pelleted by centrifugation with 8000 rcf, the supernatant discarded and the beads washed once with PBS. The OVA-biotin was eluted by adding 30 μl lysis buffer and 10 μl 4x Laemmli buffer in combination with heating for 10 min at 50 °C. Subsequently, the beads were spun down with 21000 rcf for 5 min and the supernatant was analyzed by western blot to determine the quantity of exported OVA. Additionally, the cell lysate was used to analyze the total amount of OVA taken up by the DCs.

3.2.5.2 Cytochrome c export assay

Sample preparation The second export assay was based on the translocation of cytochrome c into the cytosol and subsequently on the induced apoptose of the cell [163]. This apoptose was detected by the staining of phosphatidylserine, which is presented on the cell surface of apoptotic cells. For this labeling, AnnexinV was used. To analyze the cytochrome c export, differently treated DCs were harvested and $0.5 \circ 10^6$ cells were plated per well in a non-treated 24-well plate in 250 μl medium. The non-treated dishes were used to avoid any stress effects on the cells during the final harvest procedure, which would lead to a high background level of phosphatidylserine at the cell surface. After 30 min of incubation, the supernatant was replaced by a reaction solution containing cytochrome c. This solution was based on PBS and included 9 mg/ml cytochrome c, 200 ng/ml OVA, 5mM glucose and 10 mM NH_4Cl. After 8 hrs, the cells were washed one time with PBS and once with AnnexinV buffer on the plate. Sequentially, the cells were harvested in 500 μl AnnexinV buffer by careful cell scraping and the well was rinsed once more with additional 500 μl AnnexinV buffer. The cells were collected in a FACS tube for the staining procedure.

Staining protocol For staining, the cell number was determined and the samples were adjusted to the same cell density. After spinning down the cells with 300 rcf for 3 min, the supernatant was discarded, the cells were taken up in 50 μl AnnexinV buffer and transferred into a new tube. Afterwards, 50 μl AnnexinV buffer, containing 5 μl AnnexinV, was intermixed carefully. Following 15 min of light protected incubation at RT, 400 μl additional AnnexinV buffer were added and the staining was detected by flow cytometry. Directly before measurement, 5 μl of Hoechst (stock concentration 100mM) were intermixed to identify corrupted or dead cells and exclude these during the analysis of the apoptosis induced by the cytochrome c export.

3.2.5.3 β-lactamase export assay

CCF4-loading in DCs As a third method to analyze the export of the antigens into the cytosol, the β-lactamase assay was used as described before by Cerbrian *et al.* [64]. Therefore, DCs were loaded with CCF4, a FRET (Fröster resonance energy transfer) substrate containing a cephalosporin-core connected to a fluorescein by a coumarin-link. This structure is sensitive to β-lactamase cleavage. For loading, differently treated DCs were harvested and plated in a 24-well plate with $0.5 \circ 10^6$ cells per well. After 60 min of incubation for cell adhesion, the cells were washed two times with EM-buffer within the well plate and the loading solution (2 µM CCF4 substrate as well as the anion transport inhibitor solutionD in EM-buffer) was added. The incubation was done light protected for 1 hr at RT. During this incubation time the FRET substrate was enriched in the cytosol of the cells.

β-lactamase export assay For analysis of the antigen translocation into the cytosol, 1 mg/ml β-lactamase and anion transport inhibitor solutionD in EM buffer were added to the CCF4-loaded DCs, after washing them twice with EM-buffer. Additionally, the proteasome inhibitor MG132 (5 µM) was intermixed. The ability of β-lactamase to splice the lactam ring inside of the chephalosporine was used to split the FRET substrate and get a fluorescence shift from 535 nm to 450 nm. This cleavage can only occur, when the β-lactamase is translocated into the cytosol, where CCF4 was concentrated. Therefore, the cells were incubated light protected for 2 hrs at 37 °C and harvested afterwards with pre-cooled EM-buffer at 4 °C by cell scraping. Subsequently, the cells were transferred into FACS tubes, washed once with PBS and analyzed by flow cytometry. The efficiency of conversion of CCF4 was determined by calculating the ratio between 450 nm and 535 nm.

3.2.6 Endosomal flow cytometry

Endosome preparation for endosomal flow cytometry For endosomal flow cytometry at least $5 \circ 10^6$ cells were plated on a 10 cm culture dish and incubated for 1 hr to enable cell adhesion and recovery. After regeneration, the DCs were fed with 500 ng/ml fluorescence-labeled OVA for 20 min. Subsequently, the supernatant was exchanged and the cells were chased for additionally 20 min, if not otherwise notified. The antigen loaded DCs were harvested by a cell scraper with 2 mM EDTA in PBS, spun down and washed once with ice cold PBS. After repeated centrifugation, the pellet was resuspended in pre-cooled homogenization buffer and the cells were carefully homogenized two times in a steel homogenizer on ice. Afterwards, the homogenate was spun down with 200 rcf for 5 min to remove big cell fragments. Finally, the supernatant was transferred into a new tube and was ready for analysis by flow cytometry or for further processing and staining.

Analysis of sec61 recruitment To analyze the sec61 recruitment by endosomal flow cytometry, BMDCs were transduced with GFP-sec61β expressing lentivirus at culture day 3 and incubated for four additional days. At culture day 7 the BMDCs were loaded with the fluorophore-labeled antigen and endosomal preparation was done as described above. For analysis of the inhibition of the sec61 recruitment by intrabodies, the DC2.4 cell line was transduced with GFP-sec61β expressing lentivirus first and expanded for one week at 37 °C. Afterwards, the cells were distributed to the single probes and additionally transduced with the different intrabody constructs. After three days of incubation for infection and construct expression, analysis were done by endosomal preparation and flow cytometry.

Immunofluorescence staining for endosomal flow cytometry For the endosomal staining the homogenate was distributed to the single staining reactions and the volume was adjusted to 200 μl per sample. To this suspension, the primary antibodies were added in 50 μl homogenization buffer with a final concentration as listed in the material part above. After 20 min of incubation at 4 °C, the secondary antibody staining was done using additional 100 μl antibody solution in homogenization buffer with a final dilution of 1:1000. After 20 min of incubation at 4 °C, 100 μl of the 4x permeabilization/fixation solution were added and the reaction was mixed well by short vortexing. The endosomes were fixed for 20 min at RT and spun down with 8000 rcf for 5 min afterwards. Finally, the pellet was resuspended in at least 200 μl homogenization buffer for analysis by flow cytometry.

Analysis of the endosomal compartments The endosomal preparations were analyzed by flow cytometry at a LSRII (BD life science). Therefore, the threshold was reduced to a minimum and FCS/SSC voltages were adjusted to localize the endosomal population in the lower left of the dot blot in a biexponential scale. The samples were measured by a low flow ratio with about 2000 events per second to circumvent the risk of two or more particle within one drop during the measurement. This avoided the analysis of two or more compartments at once, which might lead to false positive samples.

3.2.7 Immunofluorescence microscopy

For immunofluorescence microscopy differently teated DCs were plated on a coverslip within a 24-well plate and incubated for 1 hr. For each well $0.1 \circ 10^6$ cells were used. After adhesion of the cells, the antigen or other substances were fed, if needed and indicated. Subsequently, the cells were carefully washed twice with PBS and fixed by IC fixation substrate for 15 min at RT. Thereafter, the cells were washed and permeabilized by perm solution (purchased from BD life science) for additional 15 min. Before antibody staining was done, the cells were blocked with 5 % serum for 1 hr. The origin of the serum was, if possible, mouse. If this was not usable because of cross-reaction with required antibodies, a serum of an appropriate species was used. The antibodies were added in perm solution with concentrations as indicated in the material part above. After 1 hr of incubation at RT, the cells were washed again with PBS and stained with the matching fluorescence-labeled secondary antibodies. This step was done light protected at RT for an additional hour. Finally, the samples were washed three times with PBS (5 min for each washing step) and incubated with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) during one washing step. After a last rinsing with pure H_2O to remove salt crystals, the cells were mounted with Fluromount and stored at 4 °C till microscopic analysis was done.

3.2.8 Statistical analysis

The analyses of the flow cytometry results were done with FlowJo. For evaluation of the fluorescence microscopy results ImageJ and Photoshop were used. The MOC and POI correlation coefficients were calculated with ImageJ. General statistical analyses were done by using ImageJ, Excel or Prism.

4 Results

4.1 The role of the ERAD machinery in the "endosome to cytosol" pathway of cross-presentation

The fact that the protein transport by the ERAD machinery has the same transport direction as the antigen translocation during cross-presentation (lumen of the ER or the endosomal compartment into the cytosol), highlights the ERAD system as an interesting target for the investigation of cross-presentation. Additionally, some components of the ERAD machinery have already been described to be involved in this presentation pathway [64, 66, 78]. Therefore, we started our analyzes by investigating the influence of two different inhibitors of the ERAD system, namely ExotoxinA (ExoA) and Eeyarestatin I (EeyI), on cross-presentation. While ExoA is described as an inhibitor of sec61 and impairs the export function of the ERAD system [78, 156], EeyI is supposed to interact with the ATPase p97 and thereby inhibits the protein transport from the ER into the cytosol [164, 165].

First we examined, whether the addition of these inhibitors has an effect on antigen presentation. Therefore, we fed the model antigen ovalbumin (OVA) to DCs and determined the efficiency of antigen presentation by the activation of OVA-specific $CD8^+$ CTLs (OT-I) or $CD4^+$ T-helper cells (OT-II). The IL-2 production measured by ELISA was used as read out. In accordance to previous observations [78] we detected a reduced activation of OVA-specific OT-I T-cells by cross-presentation in presence of ExoA (Fig. 4.1 A), while the OT-II T-cell activity was unaffected (Fig. 4.1 B). In order to exclude other unspecific effects, like for example different activation states of the DCs or different availability of fitting MHC-I molecules in presence or absence of the inhibitor, we loaded a fully processed MHC-I OVA peptide (SIINFEKL) on MHC-I molecules. Because this peptide can displace already loaded peptides on fitting MHC-I complexes, its presentation is independent of any processing.

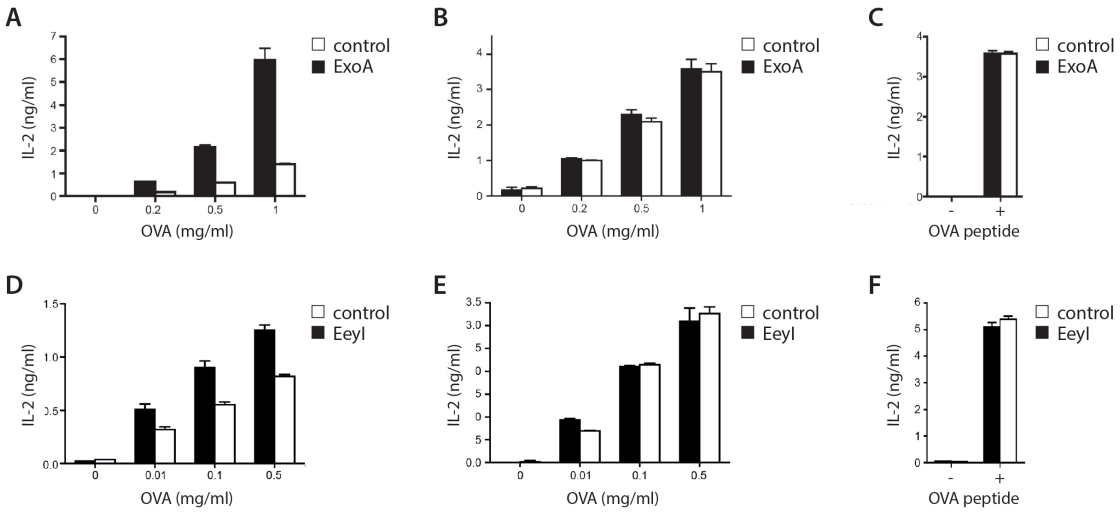

Figure 4.1: ***Inhibition of the ERAD machinery specifically impairs cross-presentation*** **A,D)** BMDCs (bone marrow-derived DCs) were incubated with the indicated amounts of OVA for 2 hrs in the presence or absence of 10 μg/ml ExoA or 3 μM EeyI. After OVA incubation the cells were washed, fixed and co-cultured with OT-I T-cells overnight. T-cell activation was measured by IL-2 ELISA. **B,E)** identical to A and D using OT-II cells. **C,F)** same as A and D using 10 nM SIINFEKL instead of OVA. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM.

Here we observed no difference between T-cell activation with or without inhibitor (Fig. 4.1 C), confirming that the amount of MHC-I molecules or the expression of co-stimulatory molecules was not affected by the inhibitor. In parallel to these results, also the inhibitor EeyI reduced cross-presentation in DCs (Fig. 4.1 D), while the MHC-II presentation as well as the T-cell activation by the presentation of the processed SIINFEKL peptide were not influenced (Fig. 4.1 E and F). Additionally, we analyzed the effect of the inhibitors ExoA and EeyI on the endogenous presentation of OVA on MHC-I molecules. After electroporation of OVA-encoding mRNA, intracellularly expressed OVA was processed and loaded on MHC-I molecules by the classical endogenous MHC-I presentation pathway. This mechanism of antigen presentation, like the MHC-II presentation, remained unaffected in the presence of both inhibitors, ExoA or EeyI (Fig. 4.2 A and B), indicating that cross-presentation might be specifically impaired by these inhibitors, while other antigen presentation pathways were not influenced.

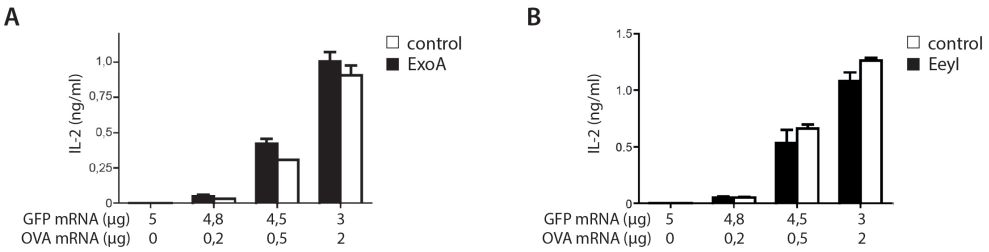

Figure 4.2: ***Inhibitors do not affect endogenous MHC-I presentation*** BMDCs were electroporated with OVA- or GFP-encoding mRNA and incubated with the inhibitors. **A)** 10 μg/ml ExoA. **B)** 3 μM EeyI. After 2 hrs incubation, the cells were fixed and co-cultured with OT-I T-cells overnight for IL-2 production. Data are represented as mean ± SEM. All graphs depict representative results of at least 3 independent experiments.

The effect on cross-presentation was also not due to a different amount of antigen available for processing, because antigen uptake was not influenced by presence of the inhibitors (Fig. 4.3). To emphasize that indeed the loading of antigen-derived peptides on MHC I molecules is impaired by the inhibition of the ERAD system, we stained OVA-treated cells with the MHC-I OVA-peptide detecting antibody 25.D1-16 and analyzed the cells by flow cytometry. A reduction of the MHC-I OVA-peptide complexes on the cell surface was visible in presence of the inhibitor ExoA or EeyI (Fig. 4.4 A and B), while the total surface expression of MHC-I molecules suitable for OVA-presentation remained unaffected. This was controlled by loading of the DCs with SIINFEKL peptide before the staining with the 25.D1-16 antibody was done (Fig. 4.4). Importantly, the MHC-I OVA-peptide staining on DCs expressing endogenous OVA was not affected by the presence of the inhibitor ExoA (Fig. 4.4 C), emphasizing that the MHC-I OVA-peptide presentation was only impaired in context of cross-presentation but unaffected in context of the endogenous MHC-I presentation of OVA. These data point out that the ERAD inhibitors might indeed specifically influence the molecular mechanism of cross-presentation.

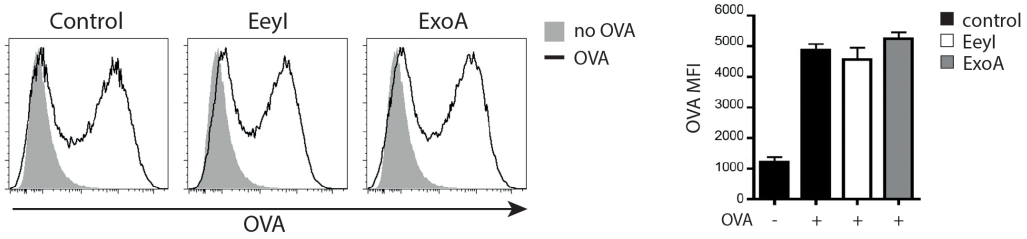

Figure 4.3: ***Antigen uptake is unaltered in the presence of ERAD inhibitors*** BMDCs were incubated with 250 ng/ml fluorophore-labeled OVA in the presence or absence of 10 μg/ml ExoA or 3 μM EeyI. OVA uptake was measured by flow cytometry. Data representative of at least 3 independent experiments are shown and are pictured as mean ± SEM. ExoA: ExotoxinA; EeyI: EeyarestatinI.

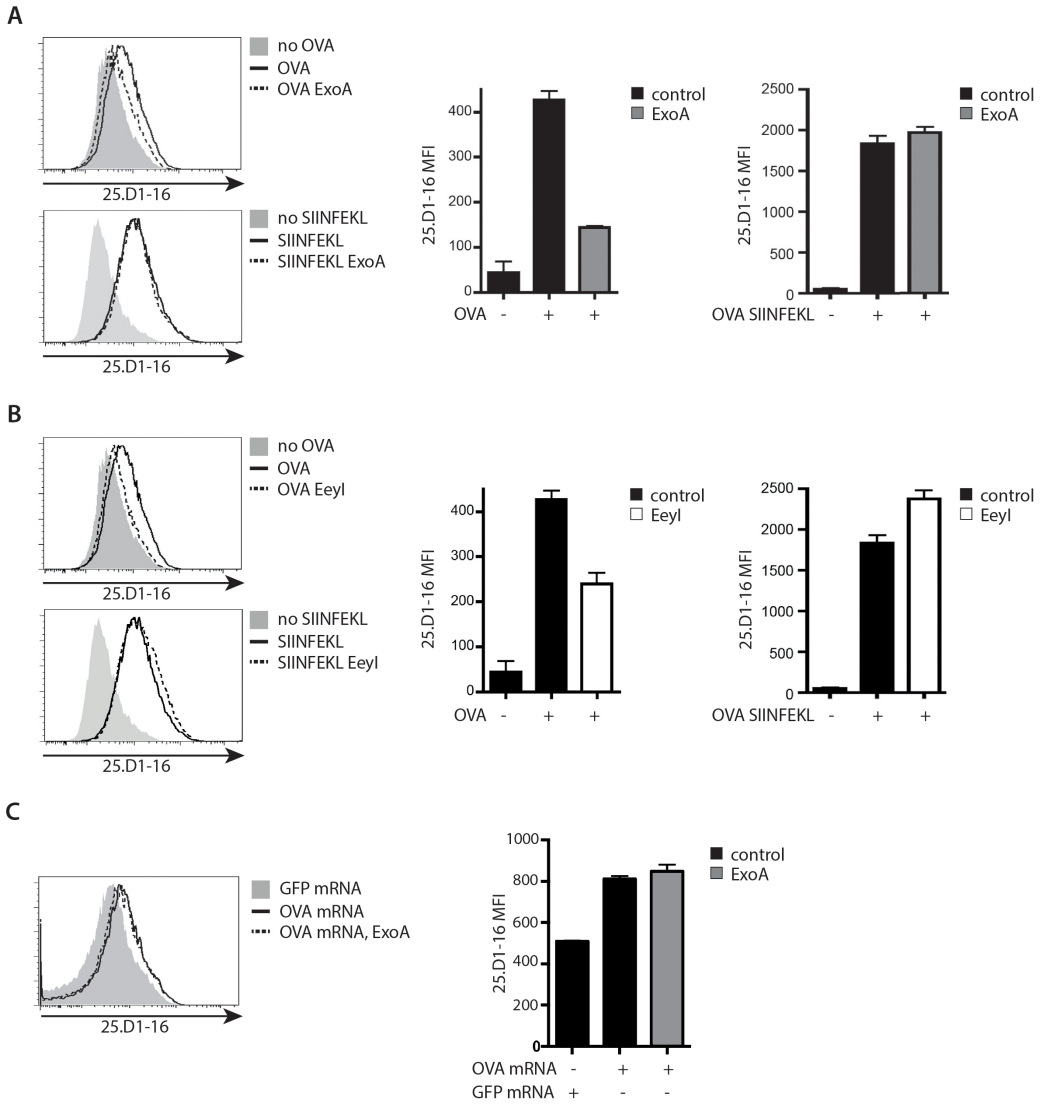

Figure 4.4: ***MHC-I presentation of OVA is reduced by ERAD inhibitors*** **A)** BMDCs were incubated with 5 mg/ml OVA or 10 nM SIINFEKL in combination with 10 μg/ml ExoA for 6 hrs. Afterwards the cells were stained with the 25.D1-16 antibody. Analysis was done by flow cytometry. **B)** Same as A) with 3 μM EeyI instead of ExoA. **C)** BMDCs were electroporated with mRNA encoding for GFP or OVA and incubated for 6 hrs in presence or absence of 10 μg/ml ExoA. Finally the cells were stained with the 25.D1-16 antibody and analyzed by flow cytometry. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM. ExoA: ExotoxinA; EeyI: Eeyarestatin I.

To clarify, whether the inhibition of cross-presentation is due to an impaired ability of the DC to export the antigen from the endosomal compartment into the cytosol, we fed biotinylated antigen (OVA) to DCs treated with inhibitor or to DCs without inhibitor. From these cells we isolated the cytosolic fraction (scheme see Fig. 4.5 A), concentrated the translocated OVA by affinity chromatography using neutravidin beads and analyzed the amount of the cytosolic antigen by western blot. We thereby registered a strong reduction of OVA in the cytosol in presence of either ExoA or EeyI, while the total amount of OVA taken up by the DCs was not influenced by the inhibitor treatment (Fig. 4.5 B and C). This confirms a putative role of the ERAD machinery during the antigen translocation from the endosomal compartments into the cytosol in context of cross-presentation.

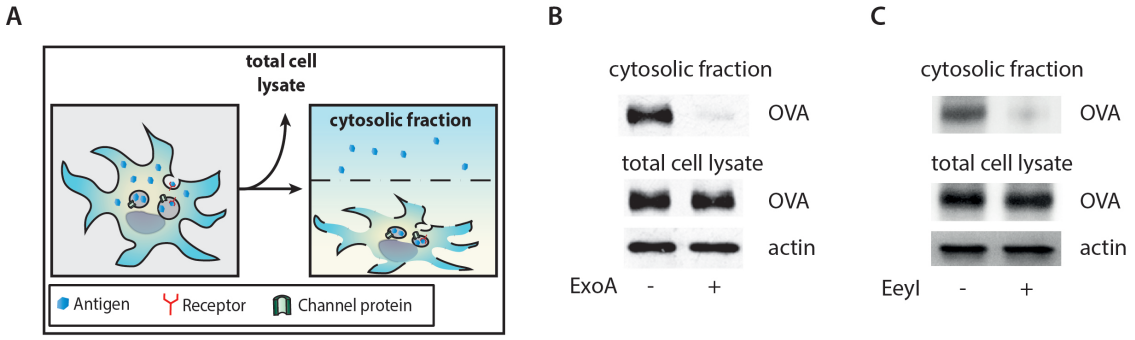

Figure 4.5: ***The antigen export is impaired by the inhibition of the ERAD machinery*** BMDCs were incubated with biotin-labeled OVA in presence of the proteasome inhibitor MG132 for 45 min and the cytosolic fraction was isolated. Afterwards, the samples were analyzed by western blot. **A)** Scheme of the experimental setup for the isolation of the cytosolic fraction of DCs **B)** Isolation of the cytosolic fraction from cells treated with 10 μg/ml ExoA **C)** Analysis of the cytosolic fraction from cells treated with 3 μM EeyI. Western blots show representative results of at least 3 independent experiments.

4.2 Sec61, but not derlin1 influences cross-presentation and antigen export into the cytosol

4.2.1 No effect of derlin1 down-regulation on antigen cross-presentation

Due to the results of the inhibitor assays, connecting the ERAD system with antigen translocation into the cytosol, we decided to analyze, which pore-building proteins of the ERAD system is involved during the intracellular antigen translocation.

To investigate potential channel proteins, we used a siRNA knock-down approach. Therefore, we first down-regulated derlin1, which plays a role in the export of misfolded proteins in the context of the ERAD machinery [137, 138]. Three days after siRNA electroporation derlin1 expression was strongly reduced in the DCs (81.5% in *siRNA A* samples and 69.8% in *siRNA B* samples; Fig. 4.6). To uncover the consequences of the knock-down of derlin1 for cross-presentation efficiency, IL-2 determination after T-cell activation was used. Although derlin1 expression was severely reduced, no effect on cross-presentation was observed (Fig. 4.7). Considering these results, the derlin1 channel protein seems not to play a major role during the antigen translocation of OVA from endosomal compartments into the cytosol.

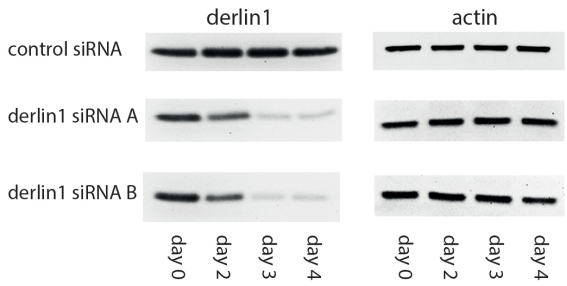

Figure 4.6: ***Down-regulation of the derlin1 protein*** BMDCs were electroporated with derlin1-specific siRNA or control siRNA. Expression was analyzed by western blot.

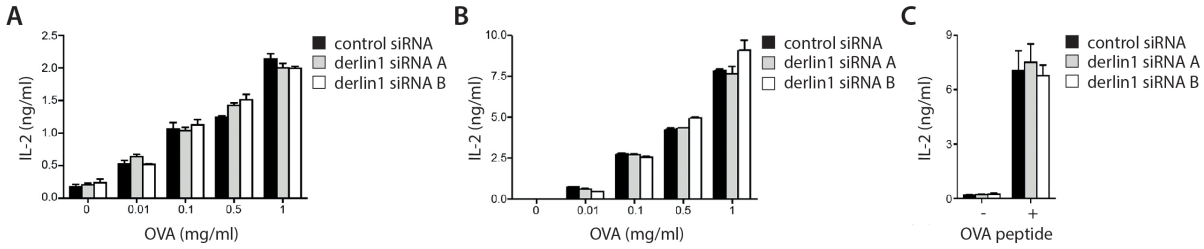

Figure 4.7: ***Down-regulation of derlin1 has no influence on cross-presentation*** BMDCs were electroporated with siRNA specific against derlin1 (two different siRNA - A and B) or control siRNA. After incubation with different OVA-concentrations for 2 hrs, antigen presentation was analyzed by IL-2 ELISA after T-cell co-culture overnight. **A)** Co-culture with OT-I T-cells. **B)** Co-culture with OT-II T-cells. **C)** Instead of OVA 10 nM MHC-I peptide SIINFEKL were loaded on BMDCs. Afterwards, these were co-cultured with OT-I T-cells overnight. Data are represented as mean ± SEM. All graphs depict representative results of at least 3 independent experiments.

4.2.2 A crucial role of sec61α in antigen cross-presentation as well as in antigen translocation into the cytosol

Next, we investigated the role of the trimeric channel protein sec61, which is, besides of its role in protein synthesis [146, 147, 148], also described as a channel protein in the context of the ERAD system [113, 150, 151, 152].
To analyze the influence of sec61 in cross-presentation, we aimed to knock-down the sec61α subunit, which builds the core of this channel protein. Because two paralogs of sec61α are available, which are nearly identical on protein level, but very different in their DNA sequence [146], we first used RT-PCR to clarify, which sec61α protein is expressed in BMDCs. The results thereby revealed that mainly the sec61α_1 protein was present in our DCs (Fig. 4.8 A). As shown by the western blot in figure 4.8 B, the siRNA knock-down of sec61α_1 reduced the total sec61α expression severely within two days, whereas only a very mild effect on the sec61α protein level was observed after sec61α_2 knock-down. Importantly, due to the nearly identical protein sequence, the western blot signal always represents the expression of both sec61α paralogs.
Analyzing the cross-presentation ability of OVA-treated DCs two days after sec61α knock-down, we observed a reduction in the cross-presentation efficiency (Fig. 4.8 C). However, at the same point in time after siRNA treatment also the MHC-II presentation was affected (Fig. 4.8 D). Additionally, the presentation of external loaded MHC-I OVA-peptides (SIINFEKL) was reduced by the sec61α knock-down (Fig. 4.8 E). These results indicate that the observed effect on cross-presentation might not be specific, but the missing of sec61α impairs the whole antigen presentation ability of DCs. This might be due to cellular stress, because of impaired protein synthesis as well as ER stress caused by a reduced ERAD function and aggregation of misfolded proteins in the ER. To circumvent these potentially stressful effects of sec61α silencing, we reduced the incubation time after siRNA electroporation to one day. At this earlier time point a significant sec61α knock-down could already be detected (Fig. 4.8 B and 4.9 A) and the cellular functions might not be severely affected.

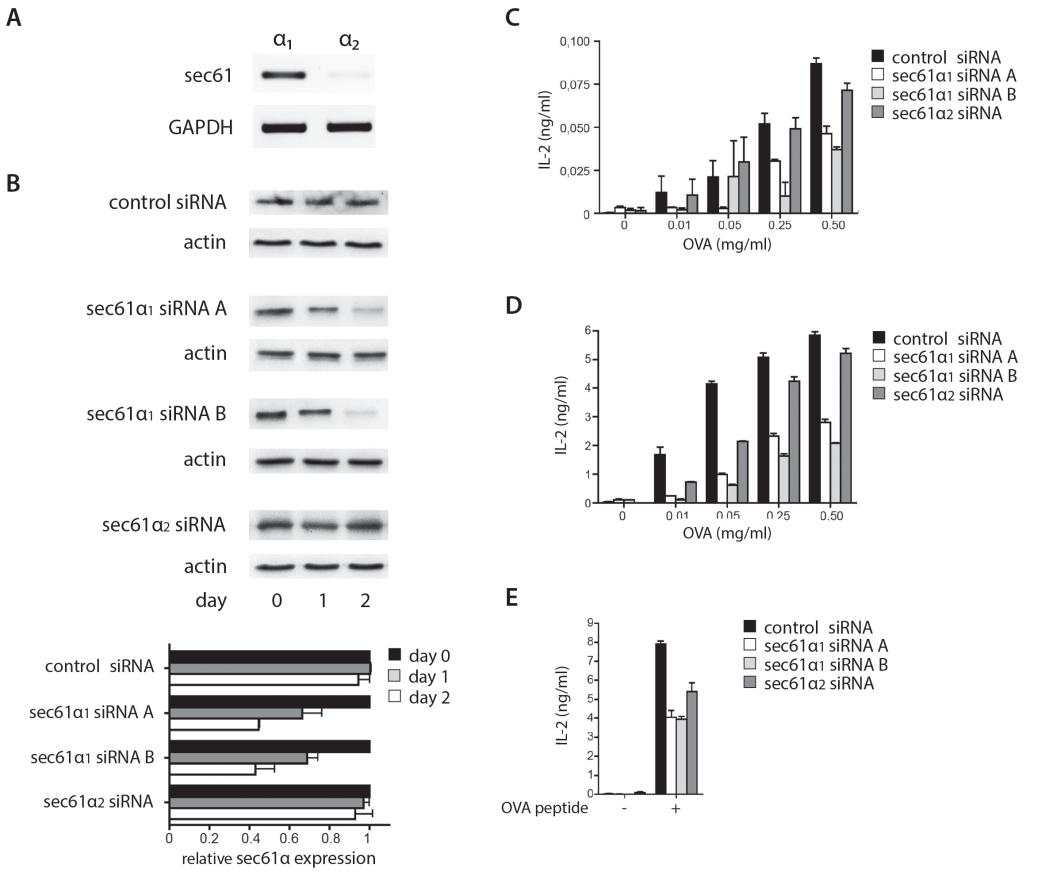

Figure 4.8: ***Strong siRNA knock-down affects antigen presentation of DCs in general*** **A)** Expression analysis of sec61α_1 and α_2 by RT-PCR on mRNA of BMDCs. **B)** Down-regulation of sec61α in BMDCs by siRNA. **C)** BMDCs were treated with siRNA against sec61α or control siRNA and cultured for 48 hrs. After sec61α knock-down, the cells were incubated with different concentrations of OVA for 2 hrs and OT-I T-cells were added. Following 18 hrs co-culture, an IL-2 ELISA was done for read out. **D)** same as C) with OT-II cells. **E)** same as C), but the cells were loaded with 10 nM SIINFEKL instead of OVA. Data are represented as mean ± SEM.

Importantly, to avoid potential stress effects during the further experiment, we immediately fixed the cells after incubation with OVA and before addition of the T-cells. Under these conditions we observed neither an effect of sec61α knock-down on MHC-II presentation (Fig. 4.9 D) nor on T-cell activation after external loading of the SIINFEKL peptide (Fig. 4.9 C). In contrast to this, the cross-presentation of the antigen OVA was still reduced one day after sec61α knock down (Fig. 4.9 B). To exclude that the MHC-I presentation pathway in general might be more stress-sensitive than the MHC-II peptide display, we analyzed the presentation of endogenously expressed OVA

on MHC-I molecules. This experiment showed that the presentation of the classical endogenous MHC-I pathway was not influenced by the sec61α knock-down after 24 hrs of incubation (Fig. 4.9 E). Additionally, the reduced cross-presentation was also not due to differences in antigen uptake after sec61α knock-down (Fig. 4.10 A).

In accordance with the low expression of sec61α_2 in DCs (Fig. 4.8 A) and western blot analysis of total sec61α expression after siRNA treatment (Fig. 4.8 B and 4.9 A), cross-presentation was not affected by sec61α_2 knock-down (Fig. 4.9).

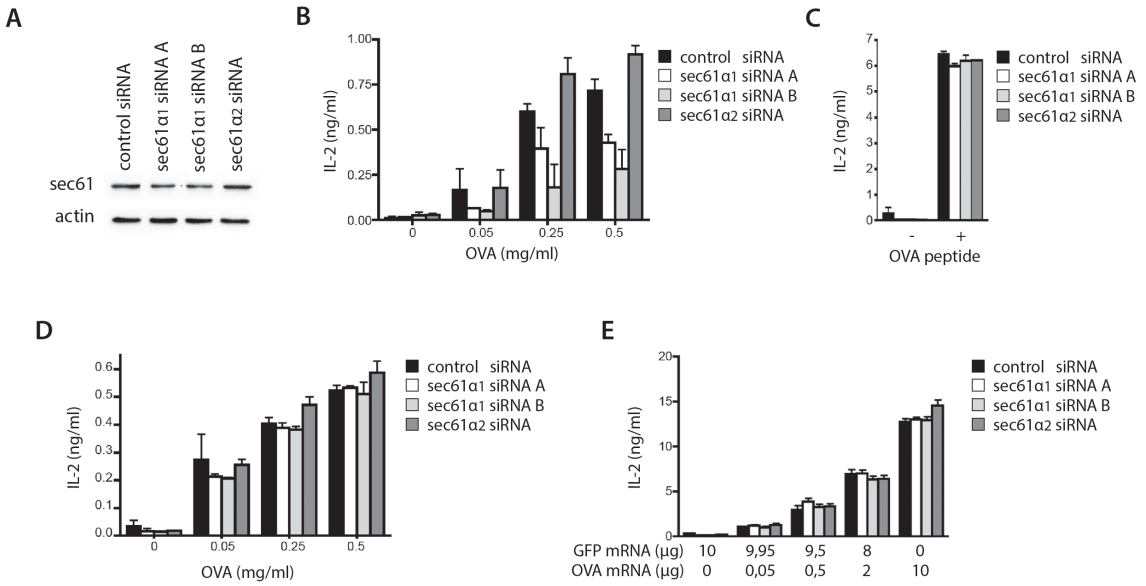

Figure 4.9: ***Reduced Sec61α_1 expression leeds to specifically impaired cross-presentation*** **A)** Sec61α knock-down 24 hrs after electroporation of siRNA. **B)** BMDCs electroporated with sec61α specific siRNA or control siRNA were cultured for 24 hrs and sequentially incubated with different OVA-concentrations. After additional 2 hrs, the cells were fixed and co-cultured with OT-I T-cells overnight. After 18 hrs, the IL-2 concentration was determined by ELISA. **C)** same as B) with 10 nM SIINFEKL instead of OVA. **D)** same as B), but OT-I T-cells were replaced by OT-II T-cells. **E)** 24 hrs after sec61α knock-down the BMDCs were electroporated with mRNA encoding for GFP or OVA. The mRNA electroporated cells were chased for 2 hrs and fixed before OT-I T-cell co-culture and IL-2 determination by ELISA was done. All graphs show representative results of at least 3 independent experiments. Data are represented as mean ± SEM.

To confirm the data gained by ELISA, we additionally stained against OVA peptide-loaded MHC-I molecules by the 25.D1-16 antibody. Subsequent flow cytometric analysis thereby revealed less cross-presentation after electroporation with sec61α_1 specific-siRNA compared to DCs electroporated with a control siRNA or with siRNA directed against sec61α_2 (Fig. 4.10 B) after 24 hrs of down-regulation. As shown by external loading

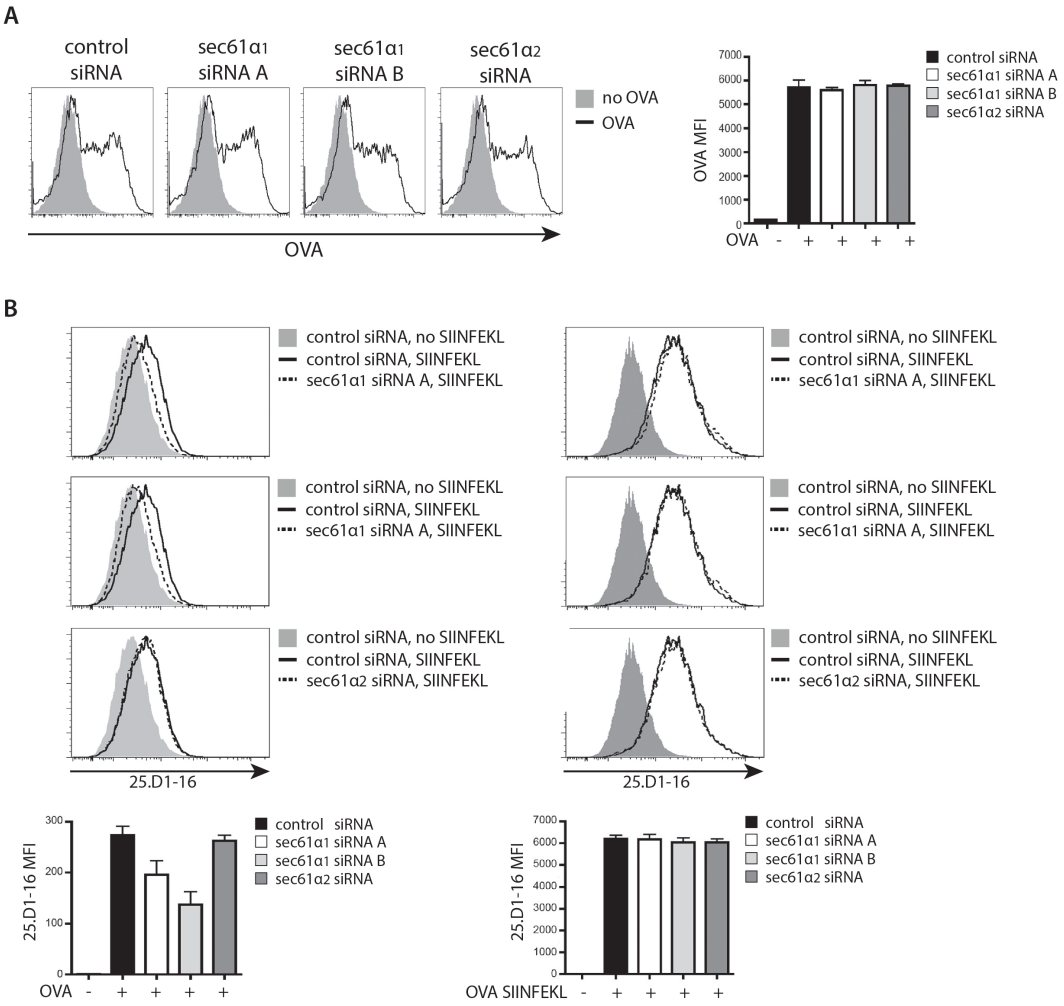

Figure 4.10: ***MHC-I OVA-peptide staining shows a reduced cross-presentation after sec61α_1 knock-down*** **A)** The BMDCs were electroporated with siRNA against sec61α or control siRNA and chased for 24 hrs. Afterwards, the cells were incubated with 250 ng/ml OVA for 15 min and analyzed by flow cytometry. **B)** 18 hrs after sec61α knock-down BMDCs were incubated for 6 hrs in presence of 5 mg/ml OVA and stained for MHC-I OVA-peptide complexes by 25.D1-16 antibody. The analysis was done by flow cytometry. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

of the SIINFEKL peptide on DCs, the amount of MHC-I molecules suitable for OVA-peptide presentation on the cell surface was not affected by siRNA treatment. These data are concordant with the specifically reduced IL-2 secretion of activated T-cells in context of cross-presentation after sec61α_1 knock-down and clearly demonstrate that the sec61 channel protein has a specific influence on this antigen cross-presentation pathway.

To investigate, whether the observed impairment of cross-presentation was due to a reduced antigen translocation into the cytosol, we separated the cytosolic fraction of OVA-treated DCs from the rest of the cell, including all antigen-containing compartments, 24 hrs after siRNA knock-down. The analysis of the cytosolic fraction of these OVA-treated DCs showed less exported antigen, when the sec61α_1 expression was reduced by siRNA knock-down (Fig. 4.11 A). To confirm these data and exclude potential artifacts during the isolation of the cytosolic fraction, like the rupture of antigen-containing endosomal compartments, we decided to use two additional assays to investigate the possibility for DCs to export antigens into the cytosol. First, we added cytochrome c to the differently treated DCs. After uptake and export from the endosomal compartment into the cytosol, cytochrome c can be localized in the cytosol and induce apoptosis (scheme shown in Fig. 4.11 B) [163]. The used experimental setup revealed that the apoptosis ratio observed in DCs was lower with a reduced sec61α expression compared to DCs treated with control siRNA. This indicated that the export of cytochrome c into the cytosol is impaired by the sec61α knock down (Fig. 4.11 C), emphasizing the importance of sec61α for intracellular antigen translocation. The second assay we conducted used the FRET (Förster resonance energy transfer) substrate CCF4, which can be split by the bacterial enzyme β-lactamase. After loading of the cells with this FRET substrate, it is concentrated in the cytoplasm of the DC and can be cleaved by β-lactamase, when the enzyme is taken up by the DC and translocated from the endosomal compartment into the cytosol. Afterwards, the resulting fluorescence shift from 450 nm to 535 nm was measured by flow cytometry. For analyzes, the ratio between cleaved and unprocessed substrate was used (scheme shown in Fig. 4.11 D) [64]. In the context of Sec61α knock-down this ratio was reduced 24 hrs after siRNA electroporation, indicating an impaired export of β-lactamase into the cytosol in absence of sec61α (Fig. 4.11 E, the presented FRET experiment was done by Dagmar Fehrenschild, bachelor student). These data confirmed the results of the first two experimental setups and again point out the necessity of sec61α for antigen translocation into the cytosol.

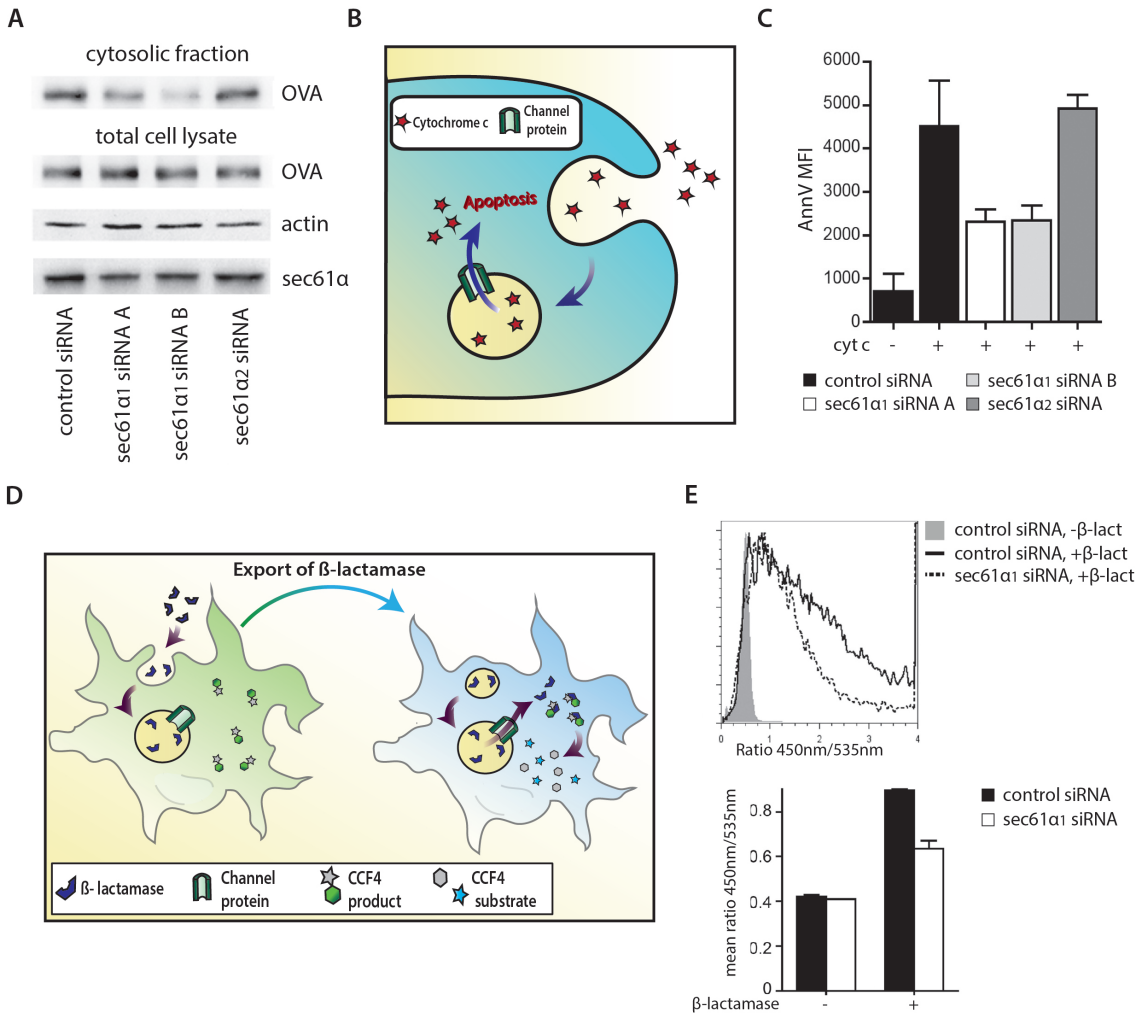

Figure 4.11: ***The antigen export into the cytosol is impaired by sec61α knock down*** **A)** BMDCs were electroporated with sec61α specific siRNA or control siRNA and incubated for 24 hrs. Subsequently, the cells were loaded with 0.5 mg/ml biotinylated OVA for 45 min in presence of the proteasome inhibitor MG132. Afterwards, the cytosolic fraction was isolated and the exported amount of OVA was determined by western blot. **B)** Scheme of the cytochrome c export assay. **C)** BMDCs treated with siRNA were cultured for 18 hrs and co-incubated with 9 mg/ml cytochrome c and 200 ng/ml OVA for additionally 8 hrs. Afterwards cells were harvested, stained by AnnexinV and analyzed by flow cytometry. **D)** Scheme of the β-lactamase export assay. **E)** siRNA treated BMDCs were incubated for 18 hrs and afterwards loaded with CCF4 for 1 hr and treated with β-lactamase for additional 2 hrs. Thereafter, the cells were harvested and analyzed by flow cytometry. Scoring was done by the ratio of cleaved FRET substrate (emission at 535 nm) to the remaining loaded FRET-substrate (emission at 450 nm). The presented β-lactamase data were generated by Dagmar Fehrenschild, bachelor student. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

4.2.3 Sec61γ is needed for efficient antigen export

To further verify the specific role of sec61 in antigen translocation, we also investigated the influence of sec61γ on cross-presentation. Sec61γ is another subunit of the sec61 protein complex and is important for the function of the channel protein [148, 149]. Like for sec61α, we used a knock-down approach to analyze the role of sec61γ in the cross-presentation machinery (Fig. 4.12 A).
The down-regulation of sec61γ expression thereby impaired the ability of DCs to cross-present OVA and subsequently reduced the activation of T-cells and IL-2 secretion, which

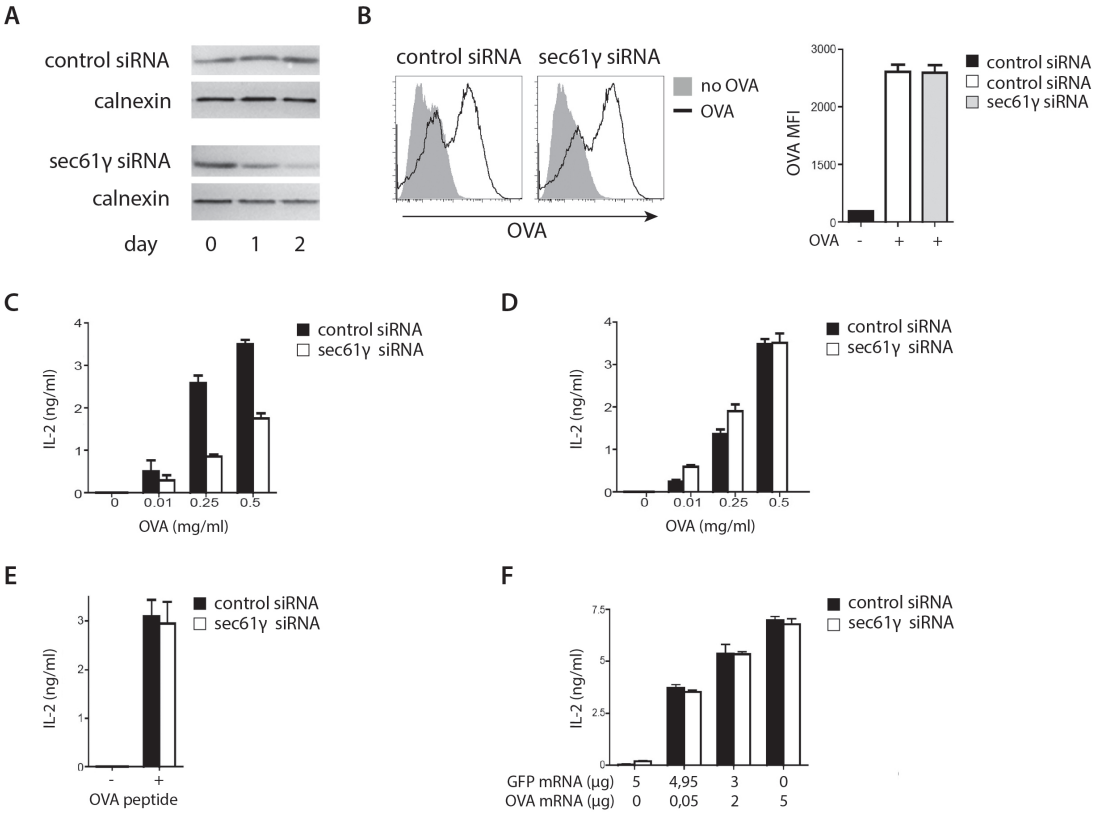

Figure 4.12: ***The knock down of the sec61γ subunit affects the antigen cross-presentation*** **A)** Western blot of the siRNA knock down of sec61γ in BMDCs. **B)** The siRNA treated BMDCs were incubated with 250 ng/ml fluorophore-labeled OVA for 15 min and analyzed by flow cytometry. **C)** After sec61γ knock down, the BMDCs were incubated with OVA for 2 hrs, fixed and co-cultured with OT-I T-cells overnight. The T-cell activation was determined by the IL-2 amount in the supernatant. **D)** same as C) with OT-II T-cells. **E)** same as C) with 10 nM SIINFEKL instead of OVA. **F)** The siRNA pretreated BMDCs were electroporated with mRNA encoding for GFP or OVA and incubated for 2 hrs. These cells were fixed and co-cultured with OT-I T-cells overnight and secreted IL-2 was determined by ELISA. All shown graphs are representative results of at least 3 independent experiments. Data are presented as mean ± SEM. MFI: mean fluorescence intensity.

was measured by ELISA (Fig. 4.12 C). On the other hand, the antigen presentation on MHC-II molecules to $CD4^+$ T-helper cells was unaffected (Fig. 4.12 D). The observed effect on cross-presentation was also not due to other unspecific effects like different activation of the DCs or different availability of suitable MHC-I molecules on the cell surface, shown by the SIINFEKL peptide loading control (Fig. 4.12 E). Additionally, the presentation of endogenously expressed OVA on MHC-I molecules was not impaired (Fig. 4.12 F). This specific effect on cross-presentation was neither caused by differences in antigen uptake, which stayed on the same level in sec61γ siRNA treated cells compared to control siRNA treated cells (Fig 4.12 B).
Importantly, analyzing the intracellular antigen export revealed a reduced amount of translocated antigen in the cytosolic fraction of DCs pretreated with sec61γ siRNA (Fig. 4.13 A). Similar to sec61α, the knock-down of sec61γ also resulted in a lower apoptosis ratio in DCs after the addition of cytochrome c.

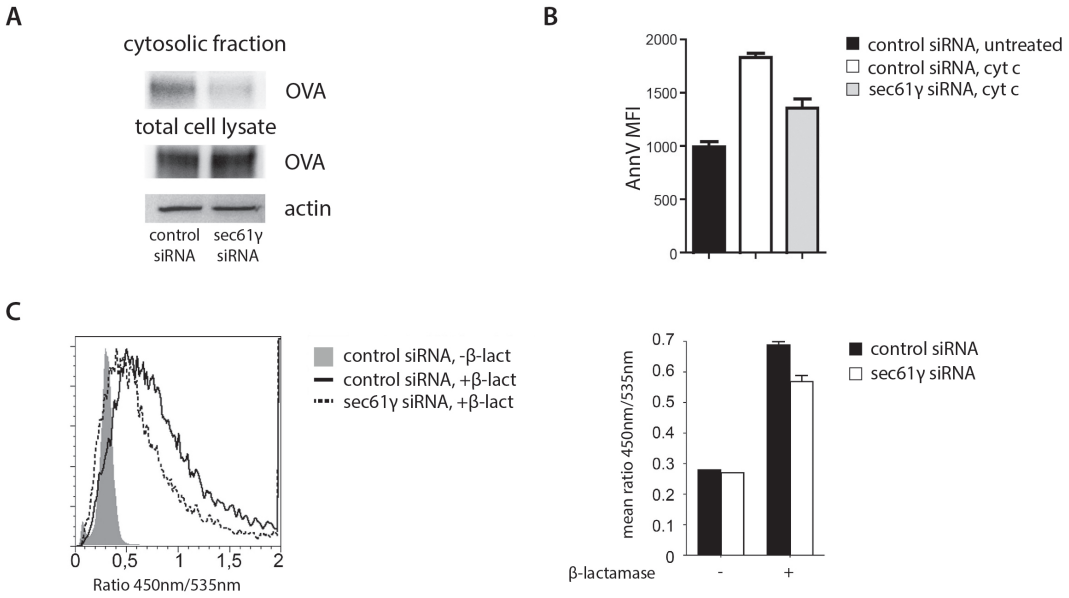

Figure 4.13: ***Sec61γ knock down reduces the antigen export*** **A)** The siRNA treated BMDCs were incubated with 0.5 mg/ml biotinylated OVA in presence of the proteasome inhibitor MG132 (5μM). After 45 min incubation, the cytosolic fraction was isolated and analyzed by western blot. **B)** Sec61γ knock-down BMDCs and control BMDCs were incubated with 9 mg/ml cytochrome c and 200 ng/ml OVA for 8 hrs, harvested and stained by AnnexinV to determine apoptosis. **C)** The siRNA treated BMDCs were loaded with CCF4 for 1 hr and co-incubated with β-lactamase for additional 2 hrs. Cells were harvested and analyzed by flow cytometry. The β-lactamase activity was determined by the ratio between the cleaved and the uncleaved FRET substrate. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

Parallel results occurred after feeding β-lactamase to DCs containing the FRET substrate CCF4. The sec61γ down-regulation reduced the conversion of the FRET substrate in the cytosol indicating a lower translocation of the β-lactamase from endosomal compartments into the cytosol of the investigated DCs (Fig. 4.13 C). These results confirmed an impaired antigen translocation into the cytosol, when sec61γ is missing (Fig. 4.13 B).

In conclusion, these observations proved that the specifically reduced cross-presentation after sec61α or sec61γ down-regulation was due to an impaired antigen export into the cytosol and emphasizes that the sec61 channel protein is involved in the antigen translocation out of endosomal compartments into the cytosol.

4.3 Sec61 is recruited to endosomal cross-presenting compartments

Considering the influence of sec61 during cross-presentation and antigen export, we wondered, whether this effect is an indirect one originating from the ER or if the sec61 channel protein is recruited to the side of antigen processing at the endosomal compartments [69].
To investigate this in a quantitative fashion we used the flow cytometry of endosomes. Therefore, we feed fluorophore-labeled OVA to the cells we aimed to analyze. After antigen uptake, we opened up the cells mechanically, isolated a crude endosomal fraction and analyzed this fraction by flow cytometry. In a previous study, we demonstrated that the resulting antigen-positive compartments (Fig. 4.14 A) are individual, intact endosomes with a size of about 500nm [157], which is the expected size of these endosomal compartments [166]. To further verify the specificity of this analytical method, we stained the crude endosomal fraction of antigen-loaded macrophages against the early endosomal marker rab5 (ras-related protein 5) or the lysosomal marker lamp1 (lysosomal-associated membrane protein 1) after different incubation periods. The staining thereby clearly showed that antigen-positive endosomal compartments (Fig. 4.14 A) mature during the incubation, resulting in a decreasing number of rab5 positive and an increasing number of lamp1 positive compartments (Fig. 4.14 B and C). This points out the possibility of this method to analyze functional and dynamic processes on endosomal compartments.

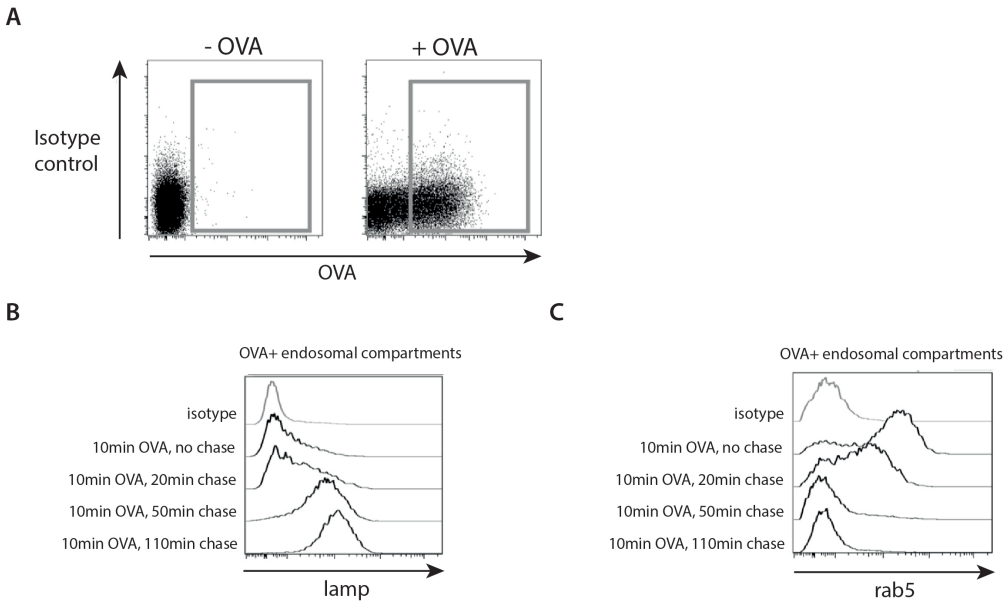

Figure 4.14: ***Antibody staining in flow cytometry of endosomes*** Bone marrow-derived macrophages were feed with 500 ng/ml fluorophore-labeled OVA for 10 min and chased for different periods of time. Afterwards, the cells were homogenized and the resulting compartments were stained with a rab5 or lamp1 antibody. **A)** An example for gating on antigen positive compartments. The gating for further analysis was always done on these OVA-positive endosomal compartments. **B)** Lamp1 staining on OVA-positive compartments. **C)** Rab5 staining on OVA-positive endosomal compartments. Data represent results of 2 independent experiments.

To subsequently investigate the potential recruitment of sec61 by flow cytometry of endosomes, we transduced DCs with a GFP-sec61β construct and treated them afterwards with fluorophore-labeled OVA. The analysis of the isolated endosomal fraction of these cells by flow cytometry revealed that the antigen-positive endosomal compartments clearly showed the presence of GFP-sec61β proteins (Fig. 4.15), pointing out that sec61 is indeed transported to these compartments and thereby is directly available to mediate the antigen translocation into the cytosol.

For further characterization of the sec61-positive antigen-containing compartments, we additionally stained the crude endosomal fraction of DCs expressing GFP-sec61β for the early endosomal marker rab5, because it was reported before that antigen-containing compartments have early endosomal characteristics [69]. A co-localization of the rab5 labeling and the GFP-sec61β signal was thereby observed (Fig. 4.16 A), emphasizing that the investigated compartments are indeed the endosomal cross-presenting compartments, which have described in previous investigations [62, 66, 69].

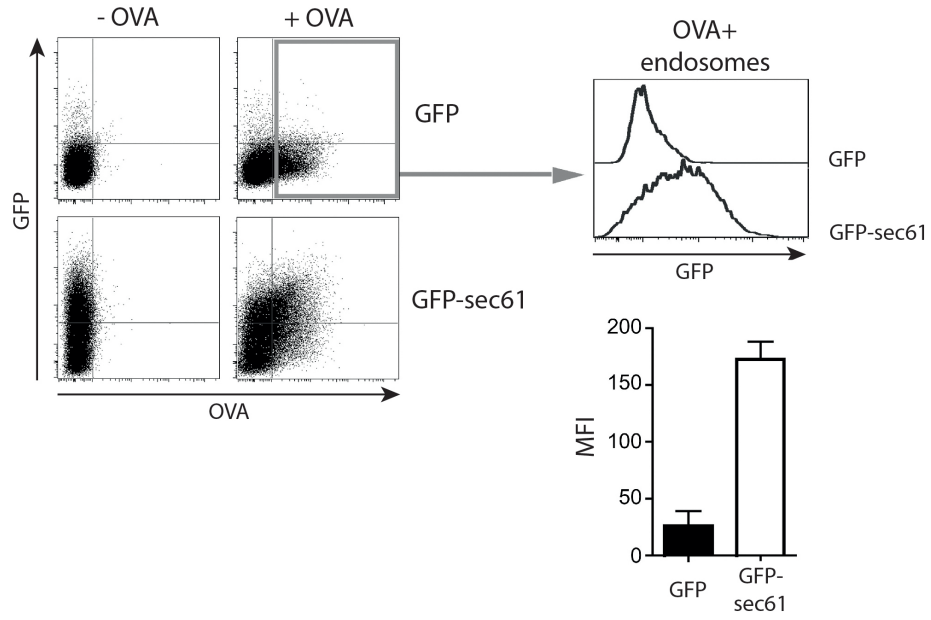

Figure 4.15: ***The sec61 channel protein is recruited to antigen-containing compartments*** BMDCs were transduced by a lentivirus encoding for GFP or a GFP-sec61β fusion protein. After 3 days, these cells were treated with 250 ng/ml fluorophore-labeled OVA for 20 min and chased for additional 20 min. The endosomal compartments were isolated by homogenization and OVA-positive endosomes (left panel) were analyzed for the presence of GFP by flow cytometry (right panel). The histograms on the right depict, as indicated, OVA-positive endosomal compartments. All shown graphs are representative results of at least 3 independent experiments. Data are presented as mean ± SEM. MFI: mean fluorescence intensity.

Interestingly, adding BrefeldinA (BrefA), which inhibits the vesicular trafficking in a cell by impairing the activation of a group of ARF (ADP-ribosylation factor) proteins [167], during antigen uptake, diminished the correlation between OVA and sec61β (Fig. 4.16 B). This additionally illustrates that the observed recruitment is no artificial membrane fusion event during the endosomal preparation. Even more it indicates that sec61 localization in endosomal compartments is not a steady state system, but needs functional vesicular trafficking during the antigen presence and is therefore an active recruitment process.
Furthermore, when we looked at derlin1 localization by flow cytometry of endosomes, we observed only derlin1 signals in OVA-negative compartments, but not in antigen-containing endosomes (Fig. 4.16 C). This is in agreement with the results of the knock-down experiments, which show no detectable influence of derlin1 on this pathway of cross-presentation (Fig. 4.7).

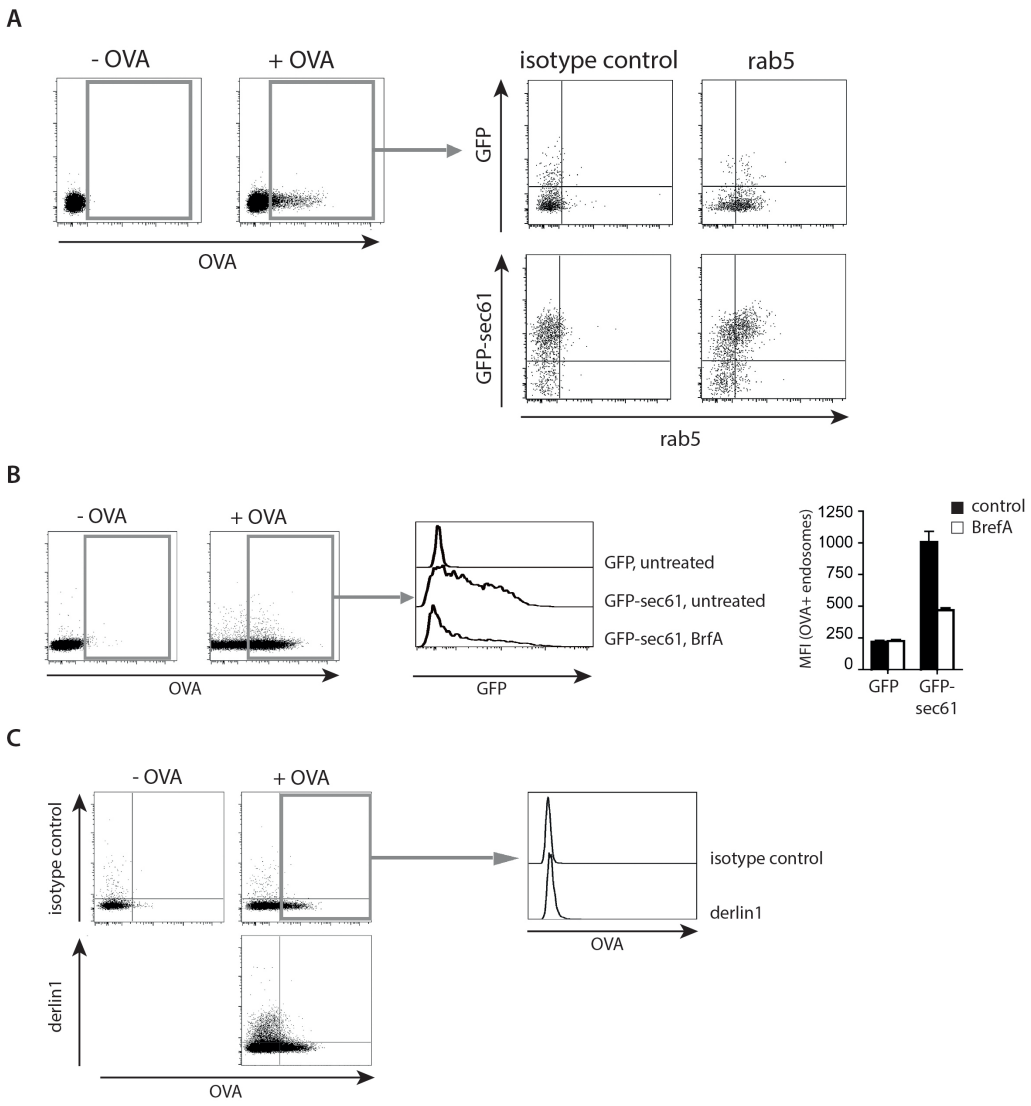

Figure 4.16: ***Sec61 is actively translocated to compartments containing the early endosomal marker rab5*** **A)** BMDCs were transduced with lentivirus encoding GFP or GFP-sec61β fusion protein. After 3 days of incubation, the cells were fed with fluorophore-labeled OVA (250 ng/ml for 20 min) and chased for additional 20 min. Compartments of BMDC were isolated by homogenization, stained with a rab5-specific antibody and analyzed by flow cytometry of endosomes. As indicated OVA-positive compartments are shown on the right side. **B)** GFP-Sec61β expressing BMDCs were incubated with 250 ng/ml fluorophore-labeled OVA with or without 10 μg/ml BrefA. After homogenization, endosomal compartments were analyzed by flow cytometry. Histograms on the right side show OVA-positive compartments. **C)** BMDCs were loaded with fluorophore-labeled antigen (250 ng/ml OVA) and incubated for 20 min. After homogenization, the compartments were stained by derlin1-specific antibodies and analyzed by flow cytometry. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

4.4 Specific inhibition of the sec61 recruitment by intrabodies

To analyze the recruitment of sec61 to antigen-containing compartments more closely as well as to definitely prove the involvement of sec61 in cross-presentation, we aimed to specifically inhibit its transport to endosomal compartments. To avoid the use of unspecific inhibitors and to target only sec61 without affecting its general function, we decided to use an intracellular antibody (also termed intrabody (IB)) to keep sec61 in the ER. IBs are intracellular expressed constructs containing the variable parts of an antibody connected by a linker sequence (Fig. 4.17 A).

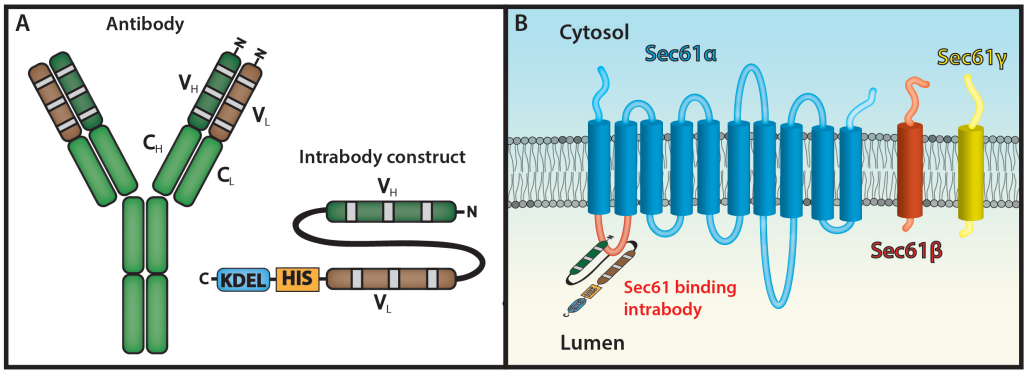

Figure 4.17: ***Generation of a sec61α specific IB*** **A)** The general structure of an IB construct compared with a classical antibody. The used IB contained an ER-localization sequence and the ER-retention signal KDEL. Additionally, a HIS-tag for the detection of the IB in western blot or fluorescence microscopy was integrated. **B)** Scheme of the sec61 channel protein complex with its three subunits α, β and γ. The targeted area of the sec61α-specific IB is highlighted in red.

This intracellular construct can be targeted against various proteins and is able to be expressed at different locations in the cell by the variation of its localization sequences. Aiming the ER-retention of sec61α we directed an sec61α-binding IB towards the ER and additionally added a KDEL-retention sequence to retain it inside of the ER (Fig. 4.17). The efficiency of this ER-retention has already been shown for other proteins before [168].

4.4.1 Generation and characterization of the sec61α-binding intrabody

To generate an IB against sec61α, a phage display targeted against a peptide originating from the first ER-internal luminal loop of the sec61α protein (Fig. 4.17 B) was done by Andrea Marschall in the laboratory of Prof. Dübel at the University of Braunschweig. In this screen an IB was isolated, which binds specifically and with a high affinity to

this peptide. To confirm this interaction also for intracellular expressed sec61α we did an immunoprecipitation with the IB construct. Therefore, we used a purified protein construct of the sec61α-specific IB. This construct was loaded on magnetic beads, added to a lysate of DCs and incubated overnight at 4°C. Afterwards, the IB-loaded beads were pulled down together with potentially interacting proteins by a magnet. Subsequently the pull down was analyzed on a coomassie gel.

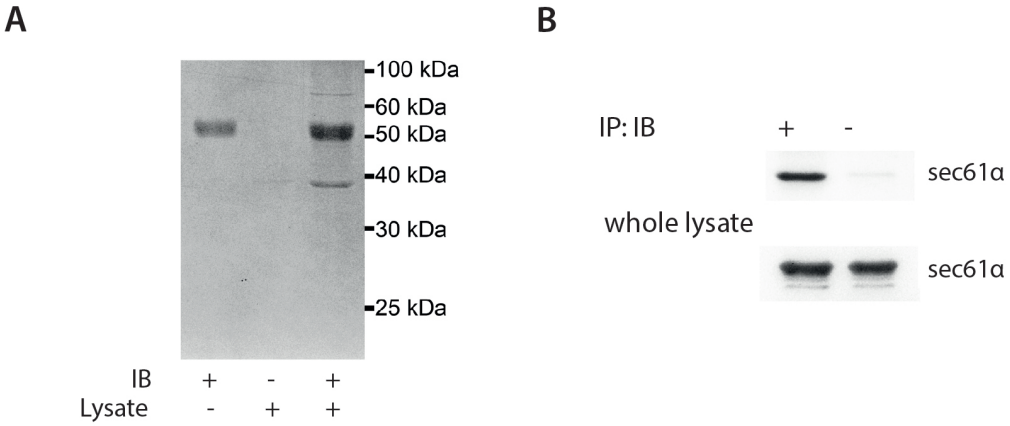

Figure 4.18: ***Characterization of the sec61α-specific intrabody*** **A)** Immunoprecipitation of BMDC lysates. The purified IB was added to the lysate and pulled down by a magnetic bead system. The samples were loaded on a SDS gel and analysis was done by coomassie staining. **B)** The immuno-precipitated samples of A) were used for western blot analysis employing a sec61α-specific antibody for staining. Gels shown here depict representative results of at least 3 independent experiments.

This gel showed, besides the IB itself and a faint band at 80 kDa, a clear band at about 40 kDa, which is the expected size of the sec61α subunit (Fig. 4.18 A). The western blot staining of the same samples with a sec61α-specific antibody proved that this band represents the sec61α protein (Fig. 4.18 B) and confirmed that the IB can also bind to the full sec61α protein, which is expressed inside of DCs, with a high specificity. To verify whether the intrabody was indeed targeted towards the ER, we performed immunofluorescence microscopy of DCs transduced with this IB. A co-localization with calnexin, an often used ER-marker protein, as well as with sec61β, another subunit of the sec61-channel complex, was detected (Fig. 4.19 A and B). These pictures clearly demonstrate that the IB is localized inside of the ER. Additionally, a non-specific control IB, which also contains a KDEL sequence, was transduced into DCs. Also this IB co-localized well with calnexin, emphasizing that the ER-retention is caused by the KDEL sequence and not by the binding to the sec61α protein (Fig. 4.19 C).

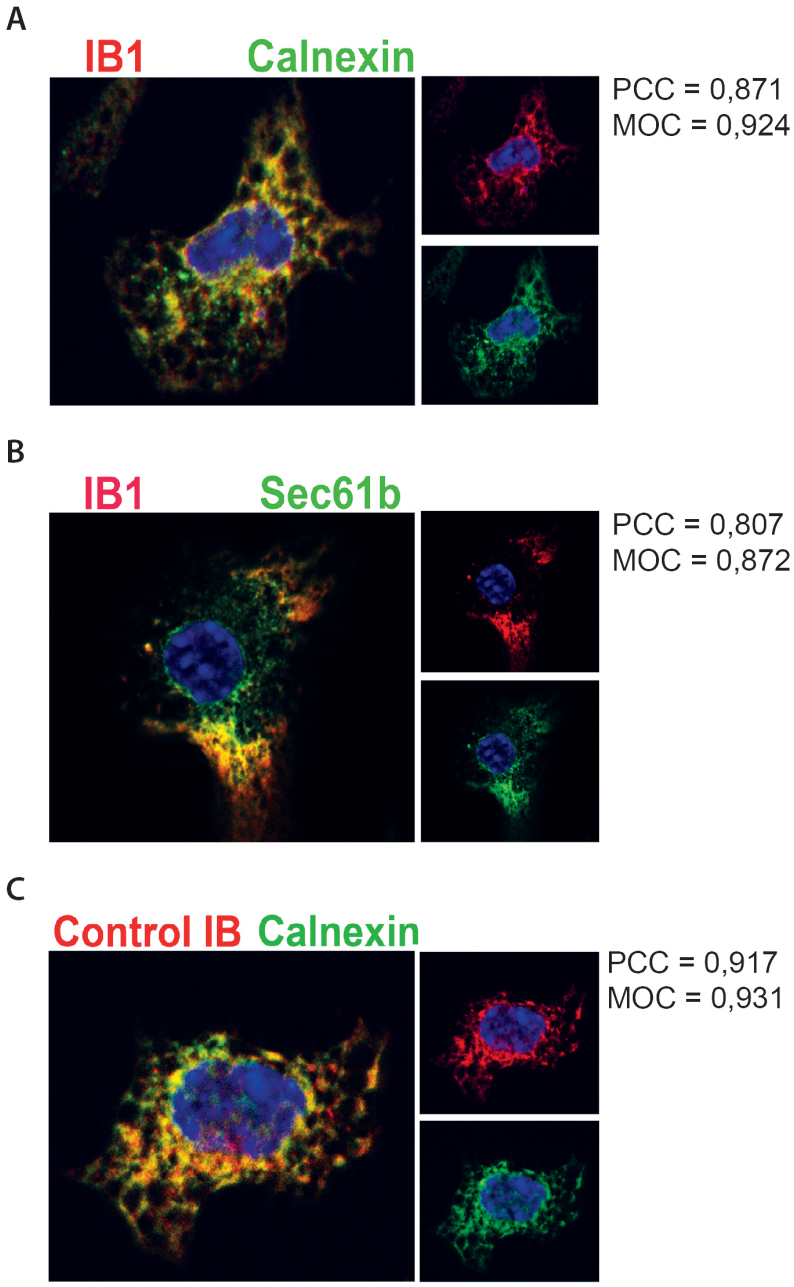

Figure 4.19: ***The sec61α-specific IB is localized in the ER*** **A)** Immunofluorescence microscopy pictures of sec61α-binding IB expression in BMDCs. The cells were stained using antibodies against the His-tag of the IB and against calnexin. **B)** Same as A) with an antibody against sec61β instead of calnexin. **C)** Immunofluorescence microscopy as in A), but the BMDCs were transduced with a control IB, which is not binding to sec61α. Nucleoli were stained with DAPI (blue). Co-localization analysis was done by Pearson correlation coefficient (PCC) and Mander's overlap coefficient (MOC).

4.4.2 The sec61α-binding intrabody does not affect the general function of sec61

Importantly, to exclude potential side effects of the interaction between the sec61 channel protein and the sec61α-binding IB, we analyzed, whether the expression of this IB alters the function of sec61 in the ER. First, we controlled the total cellular expression level of sec61α in presence of the sec61α-binding IB or the control IB by western blot analysis. The IB interaction with the sec61 channel thereby neither reduced the expression of sec61α in HEK293T cells nor in BMDCs (Fig. 4.20).

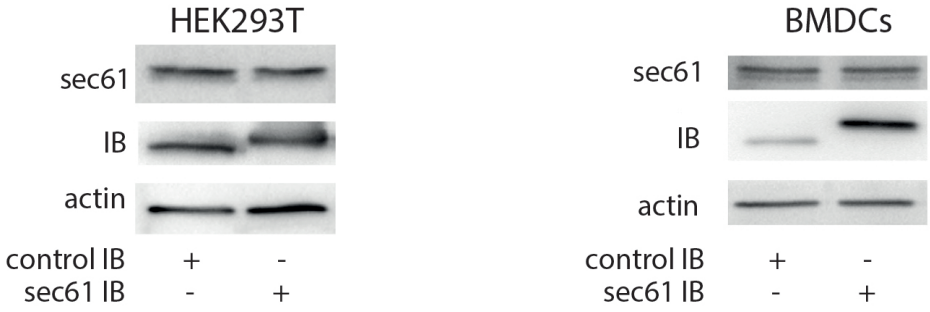

Figure 4.20: ***Sec61α-specific intrabody-binding does not impair the sec61α expression in the cell*** HEK293T cells or BMDCs were transduced by lentivirus expressing the sec61α-binding IB or the control IB and incubated for 3 days. Analysis of sec61α expression was done by western blot. Shown western blots are representative of at least 3 independent experiments.

Next we investigated, whether the general function of sec61 is affected by the IB expression. To this end, a HEK cell line expressing a cleaved fluorescent protein (Venus) was used to analyze the functionality of sec61 in the ER [162]. In this cell line one half of the Venus protein (ZV2) is expressed in the cytosol, while the other half (ddV1Z) is localized in the ER. Importantly, the fluorescence only occurs after reunion, if the ddV1Z part is efficiently expressed as well as glycosylated in the ER and subsequently deglycosylated before the export through the ERAD system is done. Thus, the missing of important components of the ERAD system, including sec61, impairs the translocation of the ER-part of the Venus protein and thereby reduces the fluorescence intensity [162]. Using this cell line we expressed the sec61α-binding IB or a control IB for 3 days. Subsequent analysis by flow cytometry showed no difference in fluorescence intensity, reflecting an unaltered function of the ERAD system, between cells containing the sec61α-binding IB and cells containing a control IB (Fig. 4.21 A). Contrary to this, the sec61-associated inhibitor ExoA as well as sec61

down-regulation resulted in an impaired Venus-protein fluorescence, confirming that a full functionality of sec61 is required for the fluorescence signaling of these cells (Fig. 4.21 B and C). This emphasizes that the expression of the IB does not affect the sec61 activity in the ER.

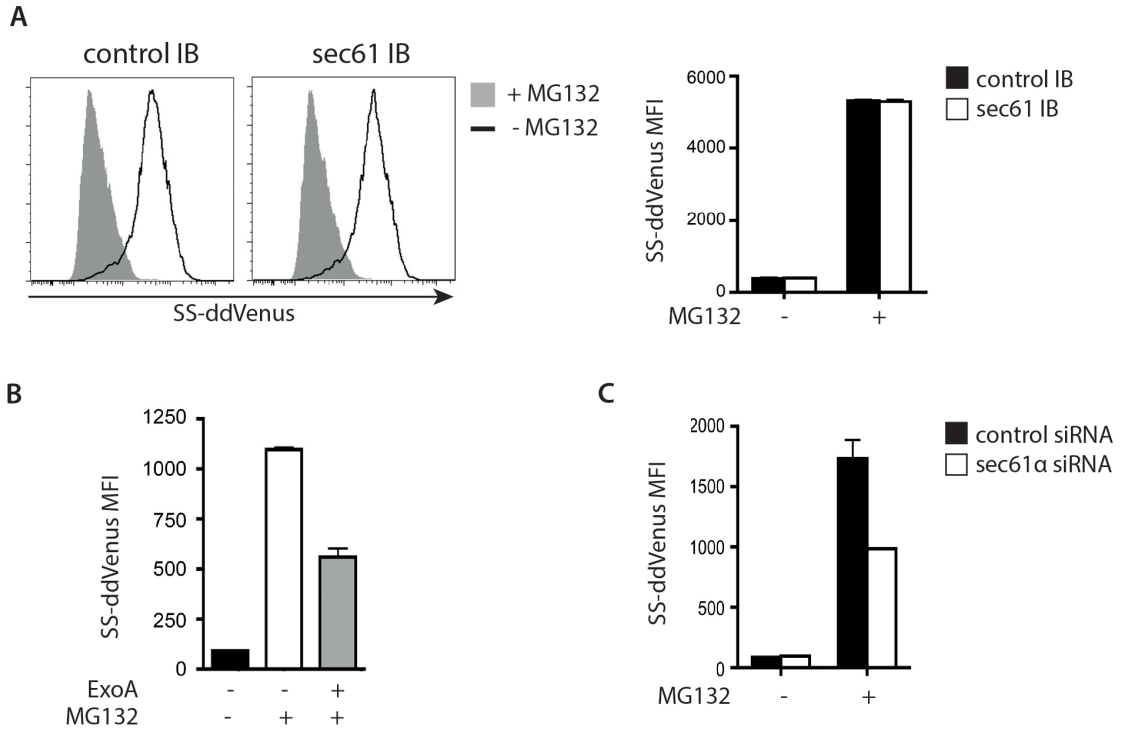

Figure 4.21: ***Sec61α-specific intrabody expression does not affect the general function of the sec61 protein*** **A)** HEK293T-Venus cells were transduced with the control IB or the sec61α-binding IB and incubated for 3 days. The proteasome inhibitor MG132 (5μM) was added for additional 6 hrs and the fluorescence of the cells was finally measured by flow cytometry. **B)** HEK293T-Venus cells were co-cultured with 10 μg/ml ExoA and 5 μM MG132 for 6 hrs and subsequently fluorescence was analyzed by flow cytometry. **C)** Sec61α-specific siRNA treated HEK293T-Venus cells were incubated for 2 days and afterwards 6 hrs in presence of MG132. Determination of the amount of reunited Venus protein was done by flow cytometry. All shown graphs are representative results of at least 3 independent experiments. Data are presented as mean ± SEM. MFI: mean fluorescence intensity. ExoA: ExotoxinA.

To fully exclude any influence of the sec61α-binding IB on the function of ERAD system in the ER, we constructed fusion proteins between an extended SIINFEKL epitope and two well-known ERAD substrates, TCRα or CD3δ [169, 170]. These proteins are translated into the ER and their final degradation depends on the ERAD-mediated translocation from the ER into the cytosol. Afterwards, the proteins are split by proteasomes and the SIINFEKL peptide becomes available for MHC-I presentation. The expression of

theses fusion proteins in DCs, transfected with the sec61α-binding IB or with a control IB, and subsequent analysis of the MHC-I SIINFEKL peptide presentation by IL2 ELISA revealed no difference in MHC-I presentation of both cell groups and therefore no difference in their ERAD function (Fig. 4.22 A and B). This again points out that the presence of the sec61α-binding IB does not influence the function of the ERAD system in the ER.

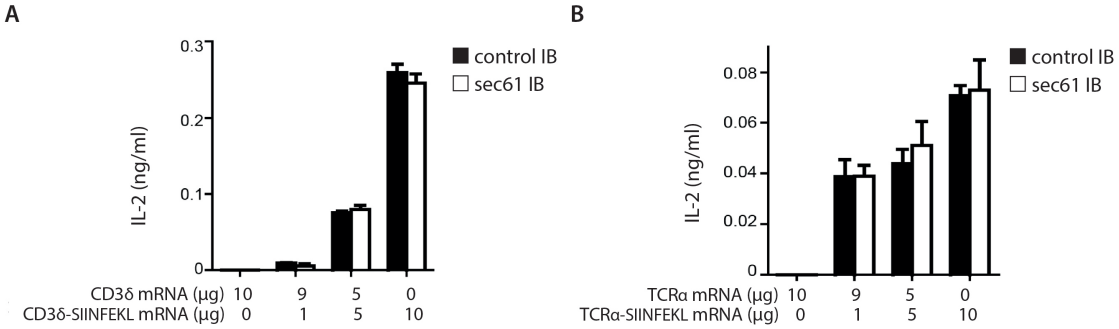

Figure 4.22: ***Expression of the sec61α-specific intrabody has no influence on the processing of different ERAD-substrates*** **A)** BMDCs were transduced with sec61α-binding IB or a control IB and incubated for additional 3 days. Afterwards the cells were harvested and electroporated with different amounts of CD3δ mRNA containing or missing a SIINFEKL coding sequence. Electroporated cells were directly plated in a 96well plate and OT-I T-cells were co-cultured overnight. After 18 hrs, the IL-2 concentration in the supernatant was determined by ELISA. **B)** Same as in A) but with TCRα mRNA instead of CD3δ mRNA. All shown graphs are representative results of 2 independent experiments. Data are presented as mean ± SEM.

4.4.3 The sec61α-binding intrabody retains sec61 specifically inside of the ER

To analyze a potential role of sec61 in cross-presentation with the help of the sec61α-binding IB, we first controlled, if the expression of this IB has an influence on the antigen uptake of these DCs. Feeding fluorophore-labeled OVA to DCs expressing sec61α-binding or control IB showed no difference in the internalized amount of antigen (Fig. 4.23 A). Additionally the localization of the antigen, which has been taken up by DCs expressing the sec61α-binding IB, was controlled by immunofluorescence microscopy. This revealed that the fluorophore-labeled antigen OVA co-localized with the early endosomal marker EEA1 as well as with transferrin, whereas no co-localization with the lysosomal protein

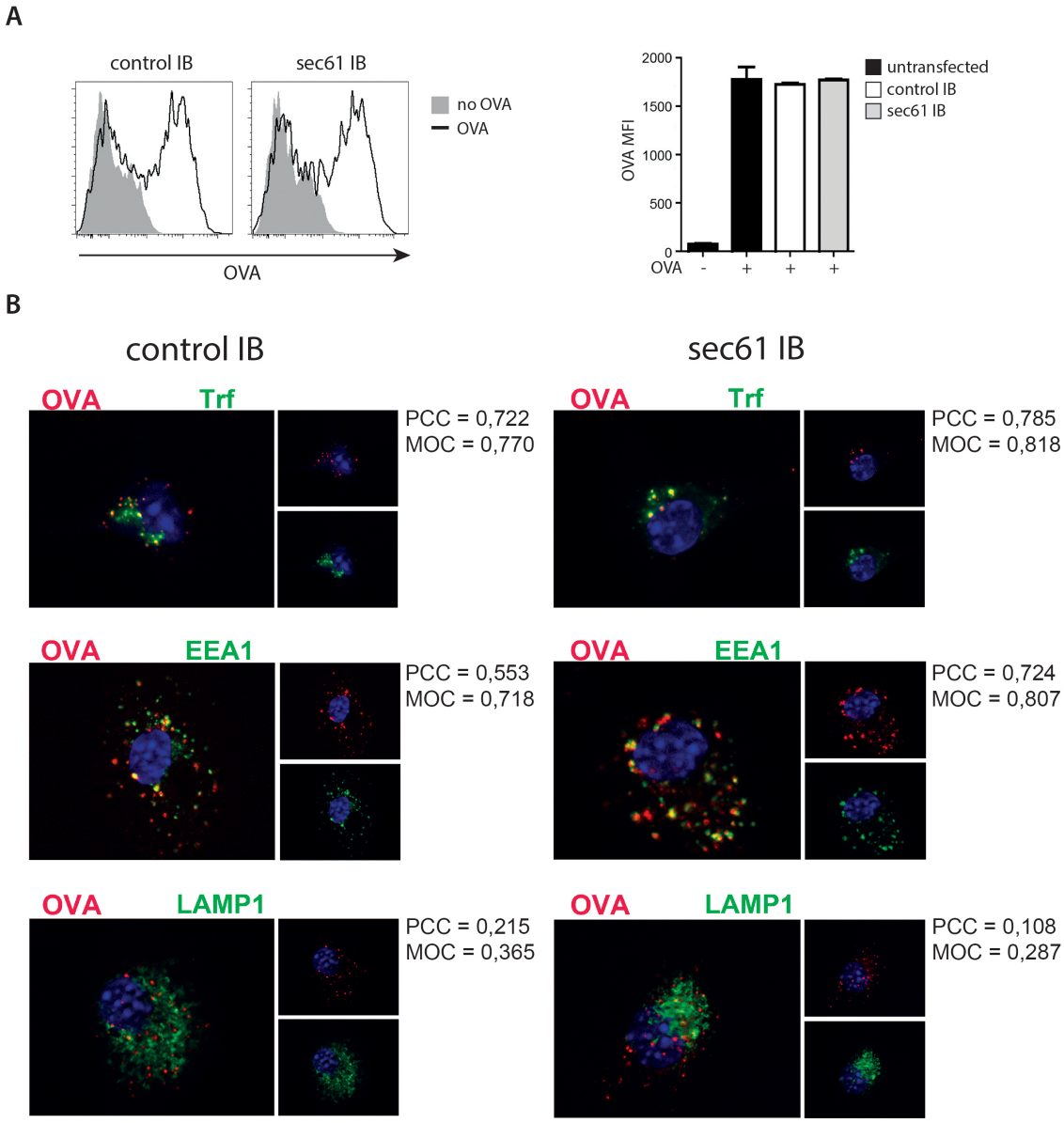

Figure 4.23: ***The sec61α-binding intrabody does not influence antigen uptake or intracellular localization*** **A)** BMDCs transduced with a sec61α-binding IB or a control IB were fed with 250 ng/ml fluorophore-labeled OVA and analyzed by flow cytometry. **B)** For immunofluorescence microscopy the cells were transduced with the IB construct and incubated for 3 days. Afterwards, the cells were fed with 250 ng/ml fluorophore-labeled OVA alone or in combination with 1 µg/ml fluorophore-labeled transferrin, incubated for 15 min and additionally chased for 10 min in fresh medium. Subsequently, the cells were fixed and the OVA-treated cells afterwards stained with antibodies against EEA1 or Lamp1. Nuclei (blue) were marked with DAPI. Co-localization was analyzed by Pearson correlation coefficient (PCC) and Mander's overlap coefficient (MOC). All shown graphs and pictures are representative results of at least 3 independent experiments. The data are presented as mean ± SEM. MFI: mean fluorescence intensity.

Lamp1 was observed. This is in agreement with the characteristics of endosomal cross-presenting compartments described before [69]. Importantly, no difference between DCs transduced with the sec61α-binding IB or with a control IB was observable (Fig. 4.23 B), emphasizing that the antigen routing also stayed untouched by the IB binding to sec61α. Next we wondered, whether the sec61α-binding IB is indeed able to retain sec61 specifically in the ER. For analysis, we transduced this IB or a control IB into GFP-Sec61β expressing DCs and loaded them again with fluorophore-labeled OVA. After preparation of a crude endosomal fraction of control IB-containing cells, a clear recruitment of GFP-Sec61β to endosomal antigen-containing compartments was detected by flow cytometric analysis. Contrary to this, the expression of the sec61α-specific IB containing the KDEL sequence strongly inhibited the transport of sec61 to these compartments (Fig. 4.24).
This demonstrated that the IB is indeed able to retain sec61α efficiently inside of the ER.

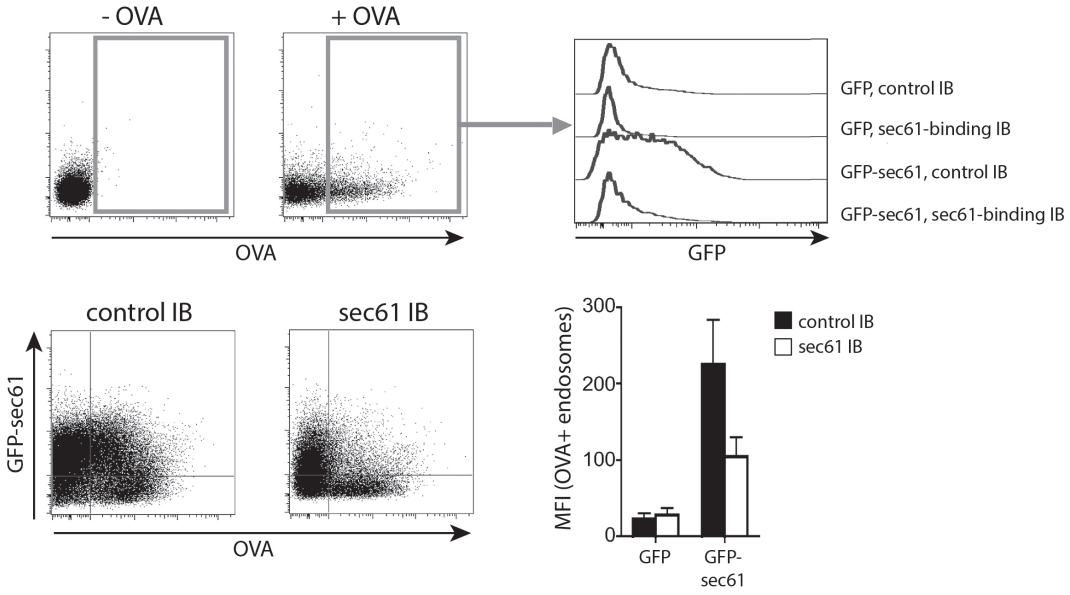

Figure 4.24: ***Sec61 recruitment to endosomal compartments is inhibited by the sec61α-specific intrabody*** DC2.4 cells were transduced with lentiviral constructs expressing GFP or GFP-sec61β and afterwards additionally infected with viruses expressing the sec61α-binding IB or a control intrabody. Afterwards, cells were treated with fluorophore-labeled OVA (250 ng/ml for 20 min) and chased for additional 20 min. Subsequently, the endosomal compartments were isolated by mechanical homogenization and analyzed by flow cytometry. Data represent results of 3 independent experiments and are presented as mean ± SEM. MFI: mean fluorescence intensity.

Subsequently, we looked at the recruitment of other components of the cross-presentation machinery to control the specificity of the sec61 ER-retention. Therefore, we stained the endosomal fraction of IB-expressing DCs against TAP as well as against calnexin, both shown to be involved in cross-presentation and present on antigen-containing compartments [62, 63, 80]. Analyses by flow cytometry pointed out that the sec61α-specific IB does not affect the recruitment of other components of the cross-presentation machinery (Fig. 4.25 A and B; experiment done by Dagmar Fehrenschild, bachelor student), emphasizing the high specificity of the sec61α-retaining IB.

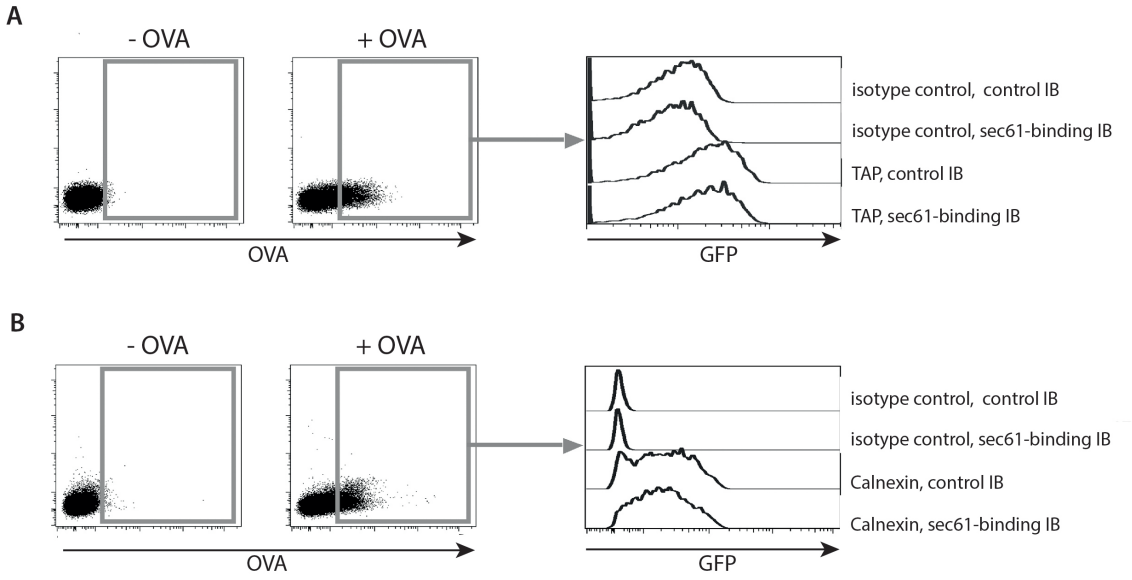

Figure 4.25: ***The recruitment of other ER-associated components of cross-presentation is not affected by the sec61α-binding IB*** **A)** BMDCs were transduced with lentivirus encoding the sec61α-binding IB or a control intrabody. After 3 days of incubation, the cells were fed with fluorophore-labeled OVA (250 ng/ml for 20 min) and chased for additional 20 min. The endosomal fraction of these BMDC was isolated by homogenization, stained with a TAP-specific antibody or an isotype antibody and analyzed by flow cytometry. As indicated OVA-positive compartments are shown on the right side. **B)** Same as A) with a Calnexin-specific antibody instead of the TAP-specific antibody. All shown graphs are representative results of at least 3 independent experiments. Data are presented as mean ± SEM. MFI: mean fluorescence intensity. Shown experiments were done by Dagmar Fehrenschild, bachelor student.

4.4.4 Sec61-retention in the ER exclusively reduces antigen cross-presentation

To analyze the importance of the sec61 recruitment to endosomal compartments for cross-presentation, DCs were transduced with the sec61-retaining IB or control IB and cross-presentation of OVA was measured by T-cell activation and detection of IL-2 secretion. Therefore, the used DCs were virally transfected at least three days before the antigen was fed for processing and presentation. Analysis showed that the retention of the sec61 channel protein in the ER specifically reduced MHC-I cross-presentation (Fig. 4.26 A). Under the same conditions MHC-II presentation exhibited no difference between sec61α-binding IB and control IB treated DCs, indicating that the MHC-II presentation pathway was not affected by sec61 retention (Fig. 4.26 B). In addition, endogenous

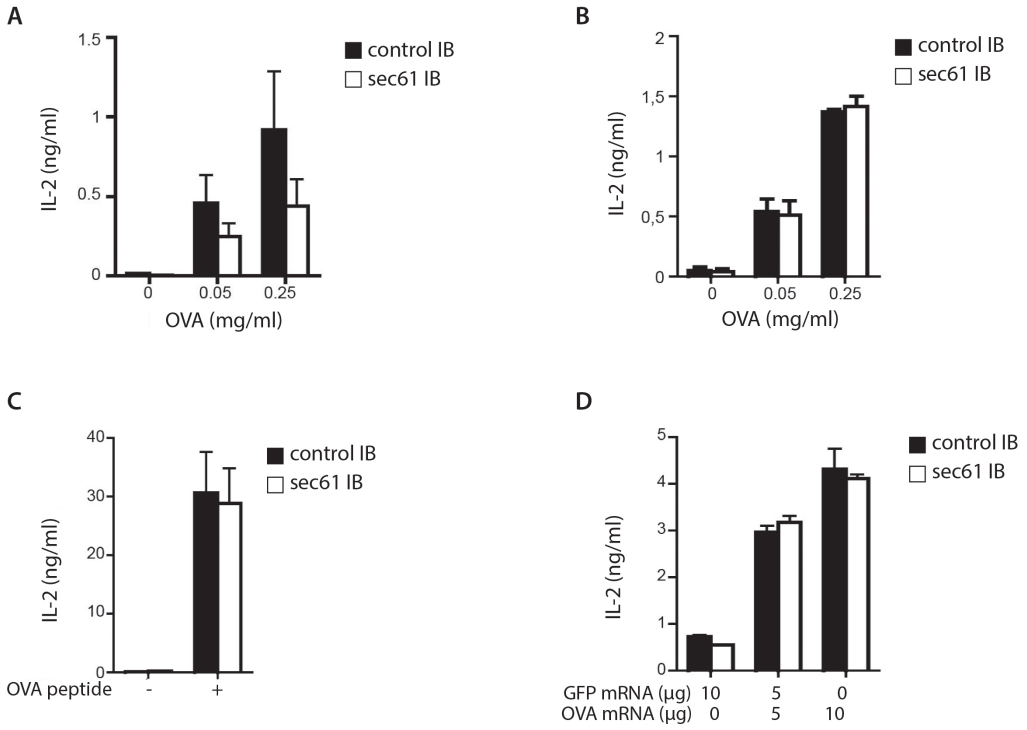

Figure 4.26: ***Sec61 ER-retention impairs specifically cross-presentation in BMDCs*** **A)** BMDCs were transduced with lentivirus encoding a sec61α-specific IB or a control IB. After 3 days of expression, BMDCs were co-cultured with different OVA-concentrations for 2 hrs, fixed and T-cells were added overnight. 18 hrs later, the IL-2 content in the supernatant was determined by ELISA. **B)** Same as in A) with OT-II cells. **C)** Same as in A) with 10nM SIINFEKL peptide instead of OVA. **D)** BMDCs expressing the sec61-specific IB or a control IB were electroporated with GFP- or OVA-encoding mRNA and incubated for 2 hrs. Cells were fixated afterwards and co-cultured with OT-I cells overnight. T-cell activation was determined by IL-2 ELISA. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM.

MHC-I presentation of OVA (Fig. 4.26 D) as well as extracellular loading of the SIINFEKL peptide (Fig 4.26 C) were not influenced by the expression of the sec61α-specific IB compared to the expression of a control IB. Besides to the analyses in primary BMDCs, the effect of the sec61α-retaining IB was confirmed in the DC2.4 cell line.
In accordance to the results in primary DCs, cross-presentation was diminished by the sec61 ER-retention (Fig. 4.27 A), while endogenous MHC-I presentation (Fig. 4.27 D), T-cell activation after external peptide loading (Fig. 4.27 C) and MHC-II presentation (Fig. 4.27 B) remained unchanged.

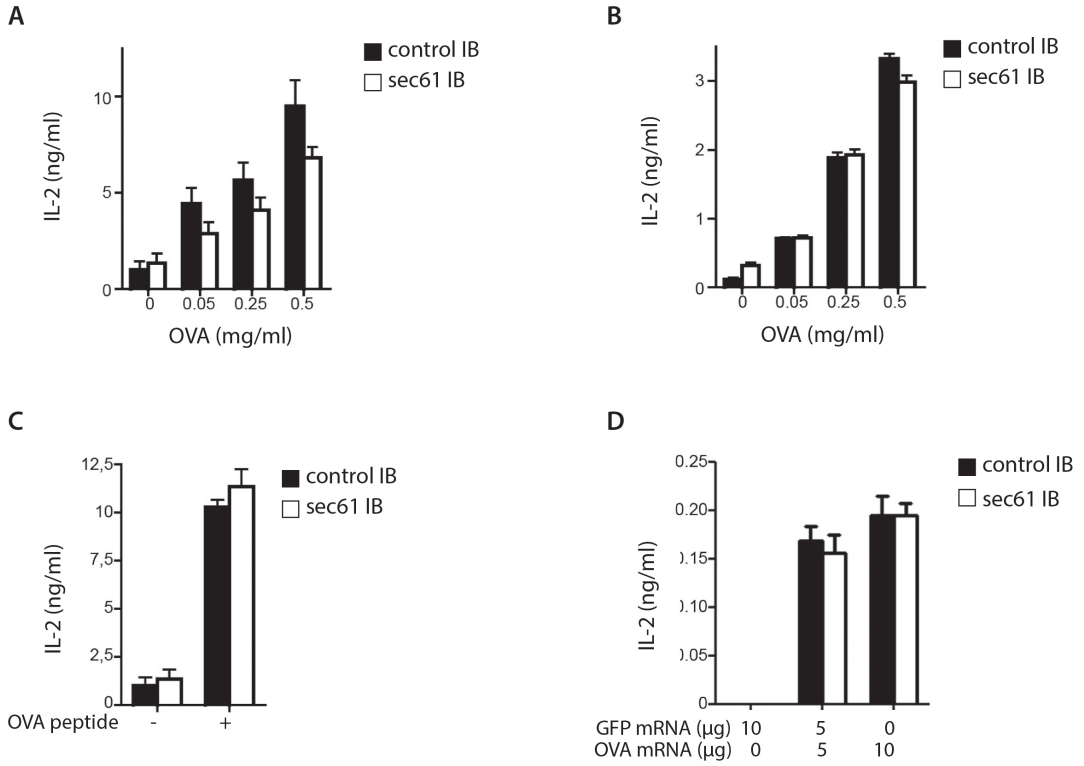

Figure 4.27: ***Sec61 ER-retention reduces antigen cross-presentation in DC2.4 cells*** **A)** DC2.4 cells were transduced with lentivirus encoding sec61α-binding IB or a control IB. After at least 3 days of expression, DC2.4 cells were co-cultured with different OVA-concentrations for 2 hrs, fixed and T-cells were added overnight. 18 hrs later, the IL-2 content in the supernatant was determined by ELISA. **B)** Same as in A) with OT-II cells. **C)** Same as in A) with 10 nM SIINFEKL peptide instead of OVA. **D)** DC2.4 cells expressing the dedicated IB were electroporated with GFP or OVA-encoding mRNA and incubated for 2 hrs. Thereafter, the cells were fixed and co-cultured with OT-I T-cells overnight. T-cell activation was determined by IL-2 ELISA. All shown graphs are representative results of at least 3 independent experiments. Data are represented as mean ± SEM.

This points out that the interaction between the IB and the sec61 channel protein neither influences the MHC-II presentation pathway nor endogenous peptide presentation at the cell surface in general. In contrast to this, the retention of the channel protein sec61 in the ER specifically suppresses cross-presentation.

4.4.5 Sec61 localization at endosomal compartments mediates antigen export

To address, whether the impaired cross-presentation, after exclusion of sec61 from antigen-containing compartments, is indeed due to a reduced antigen translocation into the cytosol, we again used three different approaches. First, DCs either expressing the sec61α-binding IB or a control IB were fed with OVA and their cytosolic fraction was isolated. In the western blot analysis a reduced amount of OVA was detected, when the IB inhibited the sec61-recruitment, compared to an efficient export in the presence of the control IB (Fig. 4.28 A).

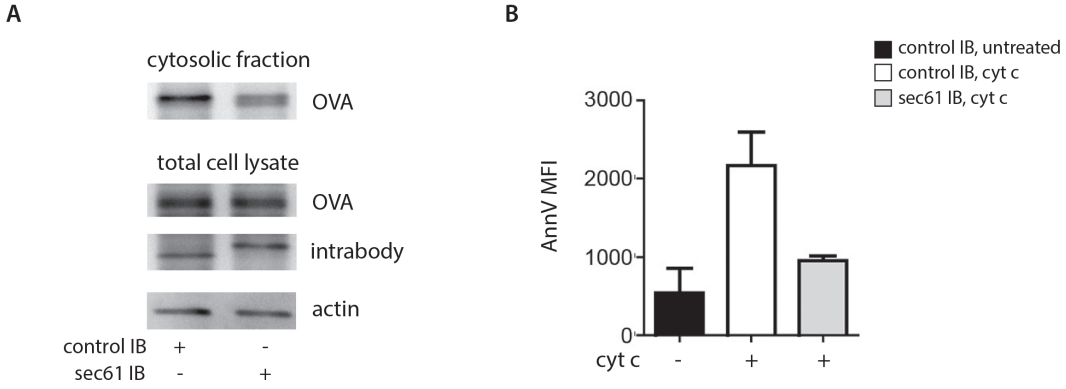

Figure 4.28: ***Expression of the sec61α-binding IB inhibits antigen export into the cytosol*** BMDCs were transduced with lentiviruses expressing sec61-specific IB or control IB. After 3 days of incubation, the antigen export was investigated. **A)** The cells were incubated with 0.5 mg/ml biotinylated OVA for 45 min in the presence of proteasome inhibitor MG132. Afterwards, the cytosolic fraction was isolated and analyzed by western blot. **B)** Infected BMDCs were incubated with 9 mg/ml cytochrome c and 200 ng/ml OVA for 8 hrs. The cells were harvested and stained with AnnexinV. Analysis was done by flow cytometry. This graphs provide representative results of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

In agreement with this, the apoptosis ratio detected by AnnexinV staining after feeding cytochrome c to DCs was reduced in the presence of the sec61α-binding IB, indicating an impaired export of the engulfed cytochrome c into the cytosol (Fig. 4.28 B). Additionally, we again loaded the FRET substrate CCF4 into the cytosol of DCs expressing the sec61α-binding IB or a control IB and fed the CCF4-splicing enzyme β-lactamase to these cells.

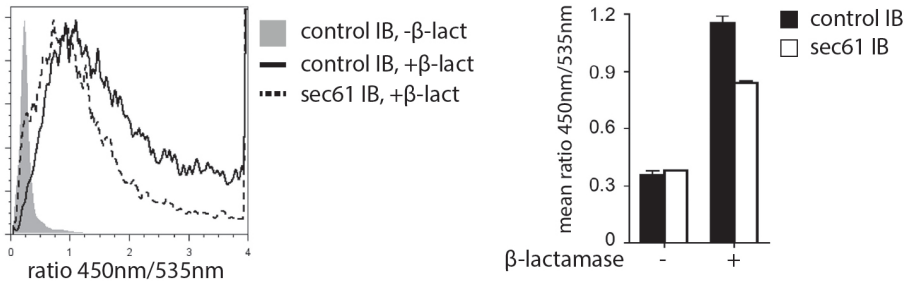

Figure 4.29: ***Sec61 ER-retention results in an impaired antigen translocation into the cytosol*** BMDCs transduced with control IB or with sec61α-binding IB were incubated for 3 days. Afterwards, IB-expressing BMDCs were loaded with CCF4 for 1 hr and subsequently fed with 1 mg/ml β-lactamase for 2 hrs. Analysis was done by flow cytometry with the correlation of spliced and not spliced CCF4-FRET substrate signal. This experiment was done by Dagmar Fehrenschild, bachelor student, and shows representative data of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

Analyses by flow cytometry thereby revealed that less CCF4 was processed in presence of the sec61α-binding IB (Fig. 4.29; the shown β-lactamase assay was done by Dagmar Fehrenschild, Bachelor student), displaying a reduced translocation of β-lactamase into the cytosol. This confirms the results of the other two export assays and demonstrates that the antigen-translocation was affected, when sec61 was missing in the antigen-containing endosomes.

These export assays clearly connect the observed reduced cross-presentation with the impaired antigen translocation into the cytosol. The fact that the translocation of three different substrates (OVA, cytochrome c and β-lactamase) was diminished by the IB-retention of sec61 emphasizes the importance of the sec61-recruitment to endosomes for the translocation of different extracellular antigens into the cytosol and subsequently for their cross-presentation on MHC-I molecules.

4.4.6 Impairment of antigen translocation by sec61α-binding IB strictly depends on ER-retention by the KDEL sequence

To conclusively prove that the ER-retention of sec61 is the reason for the reduced antigen translocation into the cytosol and subsequently cross-presentation, we used a construct of the sec61α-binding IB missing the KDEL sequence (IBΔKDEL). Expressing this construct in DCs revealed in immunofluorescence microscopy again a colocalization with calnexin as well as with sec61β (Fig. 4.30 A and B). Additionally, because of the lacking KDEL-retention sequence, the IB was, besides its ER localization, also detected

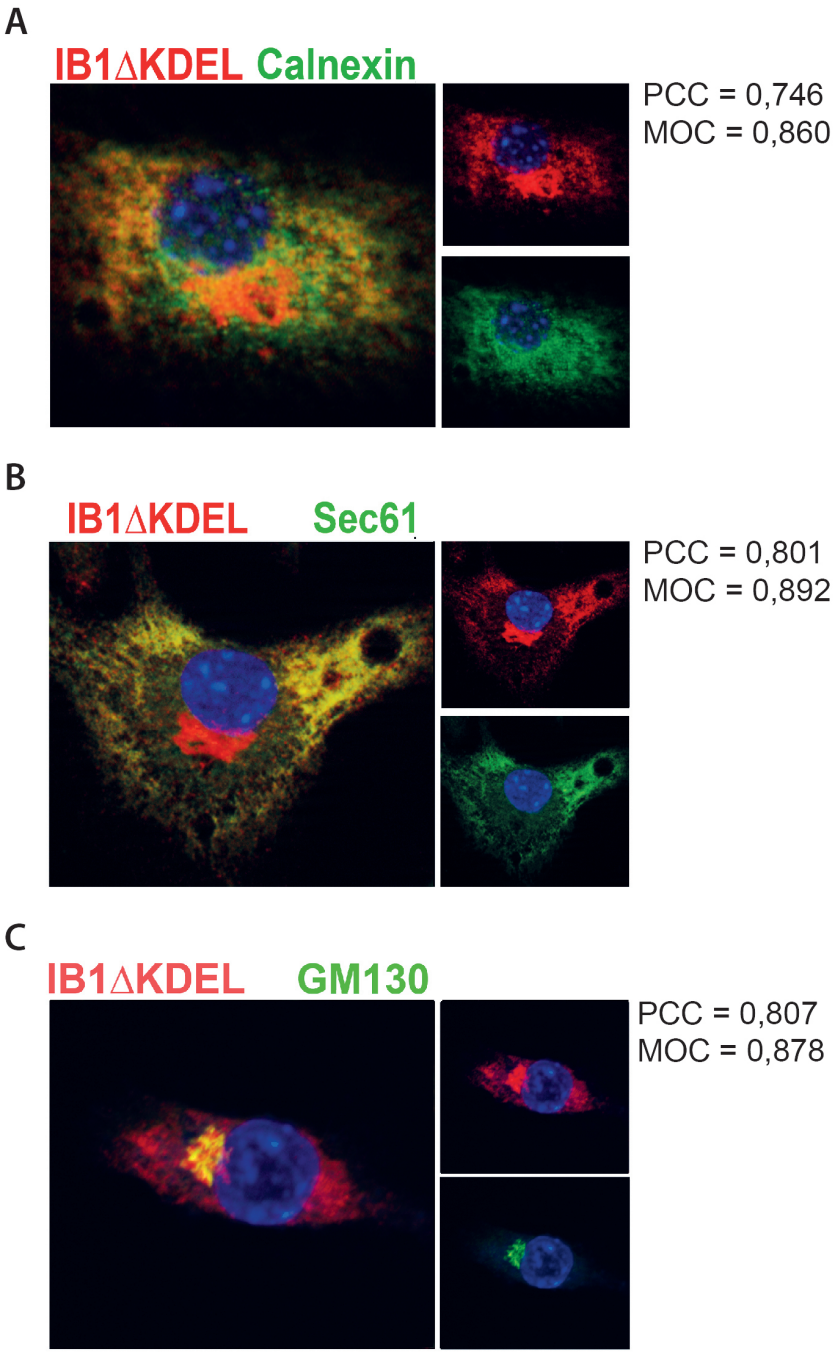

Figure 4.30: ***Intracellular localization of sec61α-specific IB missing the KDEL sequence*** **A)** Immunofluorescence microscopy pictures of sec61α-binding IBΔKDEL expression in BMDCs. The cells were stained using antibodies against the IB and calnexin. **B)** Same as A) with an antibody against sec61β instead of calnexin. **C)** Immunofluorescence microscopy like in A), but with golgi-marking GM130 antibody. Nucleoli were stained with DAPI (blue). Co-localization analysis was done by Pearson correlation coefficient (PCC) and Mander's overlap coefficient (MOC).

in another compartment of the cell. Further microscopic analysis showed that this compartment represents the golgi apparatus, which needs to be passed before protein secretion occurs (Fig. 4.30 C). Importantly, when the KDEL-missing sec61α-binding IB was expressed in DCs and cross-presentation was analyzed, no difference to the control IB was detectable (Fig. 4.31 A), although the sec61α-binding IBΔKDEL was present in the ER and able to interact with sec61α (Fig. 4.30 B). Additionally, the expression of IBΔKDEL did not alter the transport of OVA, cytochrome c or β-lactamase into the cytoplasm (Fig. 4.31 B-D).

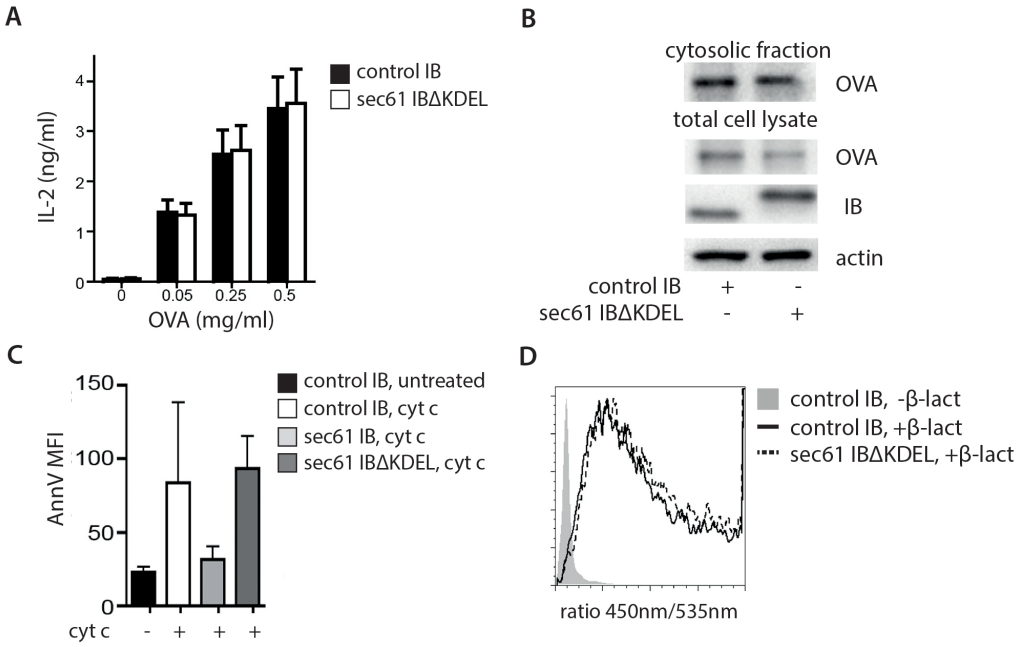

Figure 4.31: ***KDEL-mediated ER-retention of sec61α is essential for the impairment of antigen translocation*** **A)** BMDCs were transduced with a sec61α-specific IBΔKDEL or a control IB. After 3 days the BMDCs were co-cultured with different amounts of OVA for 2 hrs, fixed and T-cells were added overnight. 18 hrs later, the IL-2 content in the supernatant was determined by ELISA. **B)** BMDCs expressing different IBs were loaded with 0.5 mg/ml biotinylated OVA for 45 min in presence of MG132. Afterwards, the cytosolic fraction was isolated and analyzed by western blot. **C)** BMDCs containing different IB constructs were co-incubated with 9 mg/ml cytochrome c and 200 ng/ml OVA for 8 hrs. Afterwards cells were harvested, stained by AnnexinV and analyzed by flow cytometry. **D)** BMDCs transduced with a sec61α-specific IBΔKDEL or a control IB were loaded with CCF4 for 1 hr and treated with β-lactamase for additional 2 hrs. Thereafter, the cells were harvested and analyzed by flow cytometry. Scoring was done by the ratio of cleaved FRET substrate (535 nm) to the remaining loaded FRET-substrate (450 nm). The presented β-lactamase data were generated by Dagmar Fehrenschild, bachelor student. All graphs depict representative results of at least 3 independent experiments and are represented as mean ± SEM. MFI: mean fluorescence intensity.

These data, in combination with the previous results, unambiguously demonstrated that the reduced antigen translocation into the cytosol as well as the reduced cross-presentation after IB expression was indeed due to the specifically impaired recruitment of sec61 towards antigen-containing cross-presenting endosomes and not caused by unspecific effects due to the presence of the sec61α-binding IB in the ER.

4.5 Sec61-recruitment depends on TRIF signaling, but not on MYD88 signaling

As indicated by the addition of BrefA before antigen uptake (Fig. 4.16 B), the efficient recruitment of sec61 is not a steady state condition. Therefore, we were also interested, which signals induce and regulate the translocation of sec61 to endosomal compartments. Because especially TLR-signaling was shown to be important for the recruitment of different components of the MHC-I loading machinery to endosomal compartments in the context of cross-presentation [62, 67, 171], we decided to investigate the role of TLRs during antigen translocation into the cytosol.
First we analyzed, whether an endotoxin stimulus was needed for the sec61 recruitment. Therefore, we fed endotoxin-containing or endotoxin-free fluorophore-labeled OVA to DCs expressing the GFP-sec61β protein. The potential endosomal localization of sec61 was measured by flow cytometry of antigen-containing endosomes as described above. While efficient sec61 recruitment occurred with endotoxin-containing OVA, the recruitment of sec61 was weak, when endotoxin-free OVA was used (Fig. 4.32 A). In addition to this, we used fluorophore-labeled transferrin, which targets the same compartments as OVA [62], but does not contain endotoxins. Also the endosomal flow cytometry of transferrin-containing endosomes revealed only a weak co-localization with the GFP-sec61β (Fig. 4.32 B). To verify these low levels of the sec61 channel protein at endosomal compartments in absence of endotoxin-containing antigens, we again transduced DC with GFP-sec61β protein and analyzed the endosomal fraction of these cells without addition of antigens or additional treatment. Staining of this crude endosome fraction against rab5, which is present on sec61 positive compartments (Fig. 4.16 A), pointed out that in the absence of OVA only a few rab5 positive endosomal compartments contained additionally the sec61 protein (Fig. 4.32 C).
This confirms the results of DCs loaded with endotoxin-free OVA as well as with transferrin and proves that a stimulus of the DCs is required for efficient translocation of sec61 from the ER to the antigen-containing compartments and that only a low number of sec61 positive endosomes is present in resting DCs.

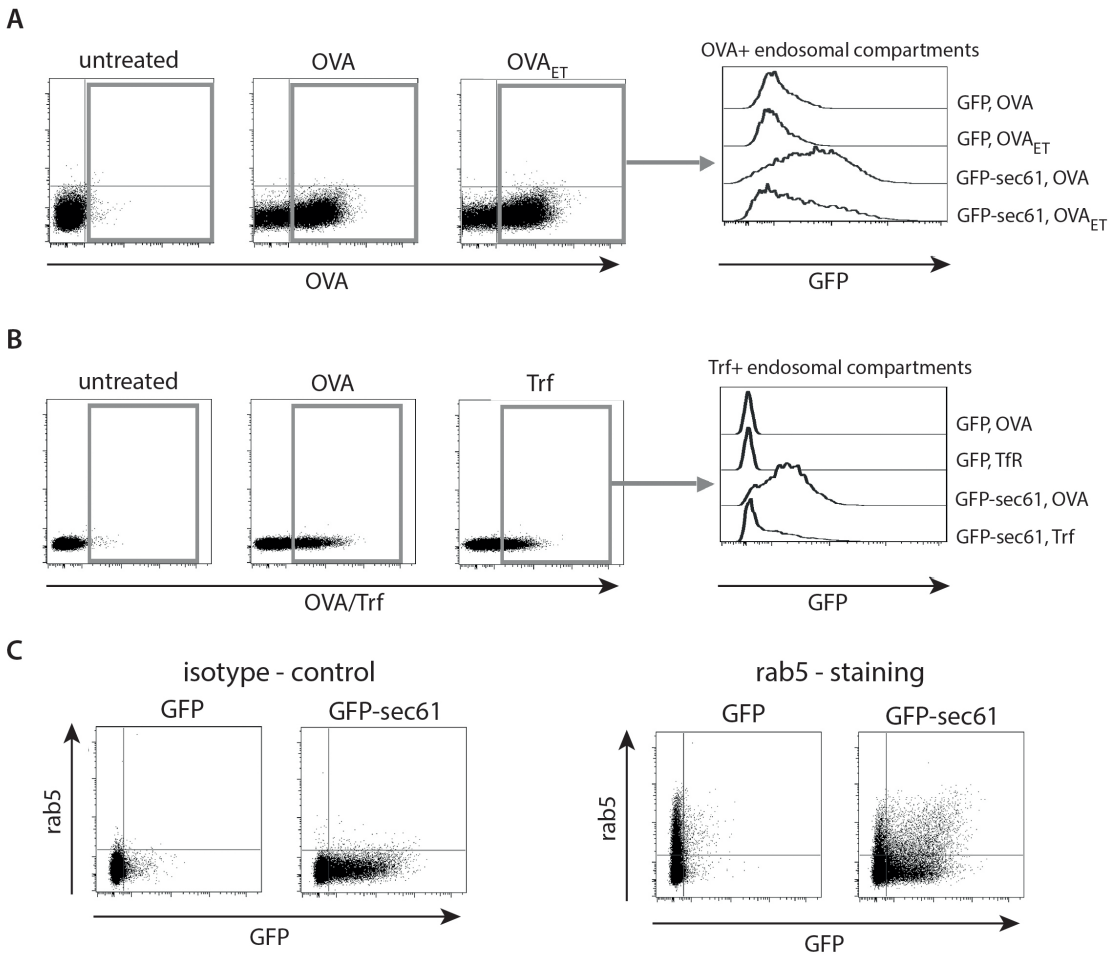

Figure 4.32: ***Efficient sec61 recruitment depends on an endotoxin stimulus*** **A)** Lentiviral transduced BMDCs expressing the GFP-sec61β fusion protein or plain GFP were incubated with 250 ng/ml fluorophore-labeled OVA with or without endotoxin for 20 min. After 20 min additional chase, the cells were opened up and the compartments were analyzed by flow cytometry. Histograms on the right side show OVA-positive endosomal compartments as indicated. **B)** DC2.4 cells transduced with lentivirus expressing GFP-sec61β or GFP were loaded with 250 ng/ml fluorophore-labeled OVA or fluorophore-labeled transferrin (Trf). Subsequently, the endosomes were isolated by mechanistically homogenization and OVA or respectively Trf positive compartments were analyzed for GFP expression by flow cytometry. **C)** GFP-sec61β or GFP expressing BMDCs were homogenized and the crude endosomal fraction was stained with rab5-detecting antibodies or an isotype control. Afterwards, the compartments were analyzed by flow cytometry. All graphs show representative results of at least 3 independent experiments. Data are represented as mean ± SEM.

4.5.1 No influence of MyD88-signaling on antigen export

To gain deeper insight into the activation signal required for the recruitment of sec61 and subsequent probably for antigen export into the cytosol and cross-presentation, the adaptor protein MyD88 was a promising first target for analysis. It is used by many different TLRs, including TLR4, which detects LPS that is present in many reagents and protein preparations (like the antigen-stocks of OVA or β-lactamase used in this study). It has also been reported that cross-presentation is affected by MyD88-deficiency [62].
Therefore, we first analyzed the involvement of MyD88-signaling in cross-presentation using an IL-2 ELISA of the supernatant of T-cells activated by wild-type or MyD88 knock-out BMDCs. We demonstrated thereby that the cross-presentation as well as the MHC-II presentation were impaired (Fig. 4.33 A and B), while endogenous presentation

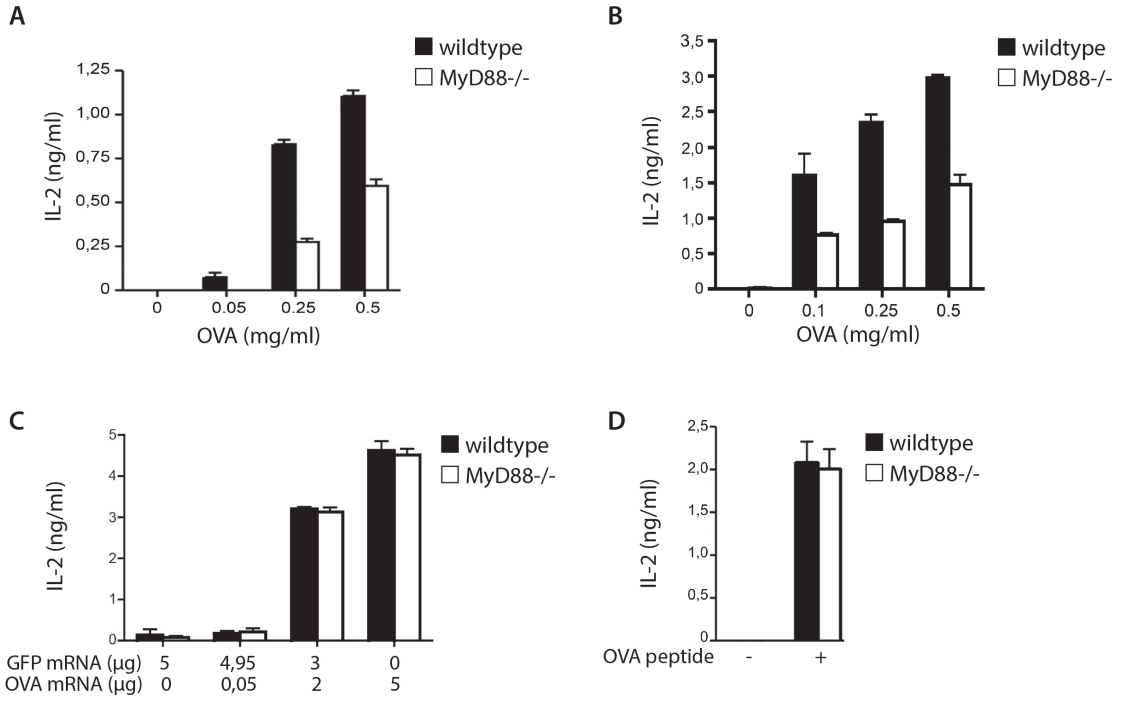

Figure 4.33: **MHC-I as well as MHC-II presentation of extracellular antigens is controlled by MyD88-signaling A)** Wild-type and MyD88-/- BMDCs were incubated with different OVA-concentrations for 2 hrs. Afterwards, the cells were washed and co-cultured with MHC-I OVA-specific T-cells overnight. Analysis was done by IL-2 ELISA of the supernatant. **B)** same as A) with OT-II cells. **C)** Wild-type or MyD88-/- BMDCs were electroporated with OVA- or GFP-encoding mRNA and incubated for 2 hrs. Subsequently, the DCs were co-cultured with OT-I cells overnight and the IL-2 amount was determined by ELISA. **D)** The same as A) with 10 nM SIINFEKL peptide instead of OVA. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM.

of OVA on MHC-I molecules (Fig. 4.33 C), the external loaded MHC-I peptide control (Fig. 4.33 D) as well as total antigen uptake (Fig. 4.34 A) were not influenced by the lack of MyD88 signaling. This indicates that the difference in cross-presentation has to be localized in the cross-presentation specific machinery.

To investigated, whether the transport of sec61 from the ER to the endosomal antigen-containing compartments and subsequently the antigen translocation into the cytosol was affected by MyD88 signaling, we used again flow cytometry of endosomes. Here we detected no difference between wild-type DCs and DCs missing the stimulus by MyD88-signaling (Fig. 4.34 B), indicating a minor role of MyD88 during sec61 protein recruitment.

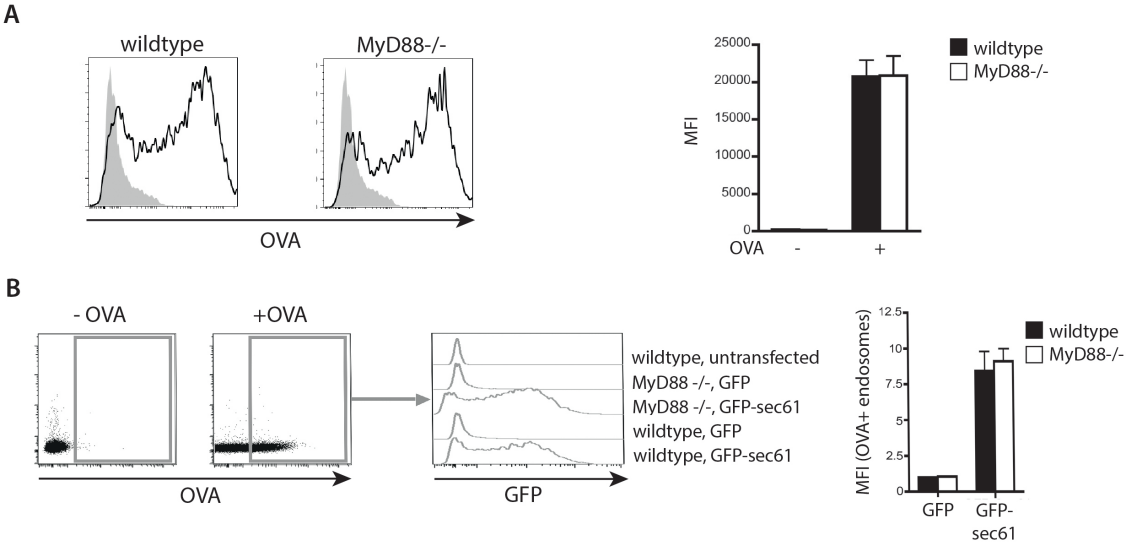

Figure 4.34: ***MyD88 signaling is not essential for the sec61 recruitment*** **A)** Wild-type as well as MyD88-/- cells were fed with 250 ng/ml OVA for 15 min and analyzed by flow cytometry. **B)** Endosomal flow cytometry of GFP-sec61β transduced BMDCs from wild-type and MyD88-/- mice after 20 min incubation with fluorophore-labeled OVA and additional 20 min chase in fresh medium. Data are represented as mean ± SEM. All graphs depict representative results of at least 3 independent experiments. MFI: mean fluorescence intensity.

Thus, it was not surprising that the amount of OVA exported into the cytosol was nearly unaffected by the knock-out of the signaling molecule MyD88 (Fig. 4.35 A). Additionally, also the other export assays, namely detection of apoptosis ratio after cytochrome c uptake (Fig. 4.35 B) as well as FRET-conversion by β-lactamase (Fig. 4.35 C) showed only a marginal or no impairment of the antigen export into the cytosol. These data lead us to the conclusion that sec61 recruitment and subsequently antigen translocation into the cytosol are not critically dependent on MyD88 signaling.

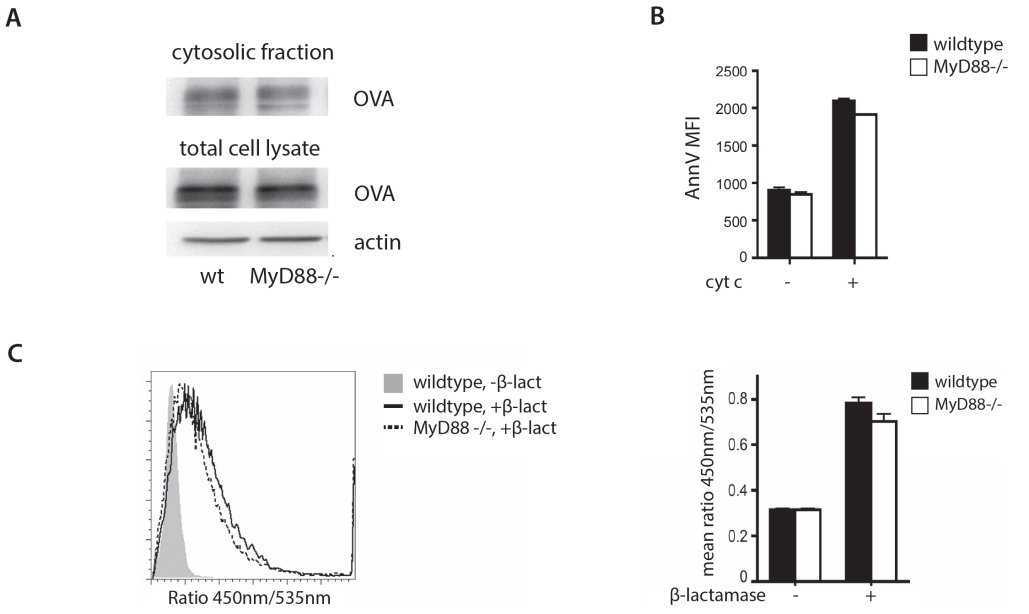

Figure 4.35: ***Antigen export is not influenced by MyD88 signaling A)*** Wild-type or MyD88-/- BMDCs were incubated with 0.5 mg/ml biotinylated OVA in presence of proteasome inhibitor MG132. After 45 min incubation, the cytosolic fraction was isolated and analyzed by western blot. **B)** MyD88-/- BMDCs as well as control cells were incubated with 9 mg/ml cytochrome c and 200 ng/ml OVA for 8 hrs, harvested and stained by AnnexinV to determine the apoptosis ratio. **C)** BMDCs from wild-type and MyD88-/- mice were loaded with CCF4 for 1 hr and co-incubated with β-lactamase for additional 2 hrs. The cells were harvested and analyzed by flow cytometry. β-lactamase export was determined by the ratio between the cleaved and the uncleaved FRET substrate. Data are represented as mean ± SEM. All graphs present the results of at least 3 independent experiments. MFI: mean fluorescence intensity.

4.5.2 TRIF-signaling induces sec61-recruitment and antigen export

A second activation pathway of TLR4 is the signaling via the adaptor protein TRIF. Therefore, we decided to analyze also the influence of this TLR pathway on antigen presentation. Using again the activation of T-cells and secreted IL-2 as indicator for efficient antigen presentation, we detected a difference between wild-type and TRIF-/- DCs in cross-presentation (Fig. 4.36 A). In contrast to MyD88-/- DCs the MHC-II presentation was not affected in TRIF-/- DCs (Fig. 4.36 B). Also endogenous MHC-I presentation (Fig. 4.36 C) and the external loading of MHC-I molecules with the SIINFEKL peptide were also not altered in TRIF-/- DCs (Fig. 4.36 D).

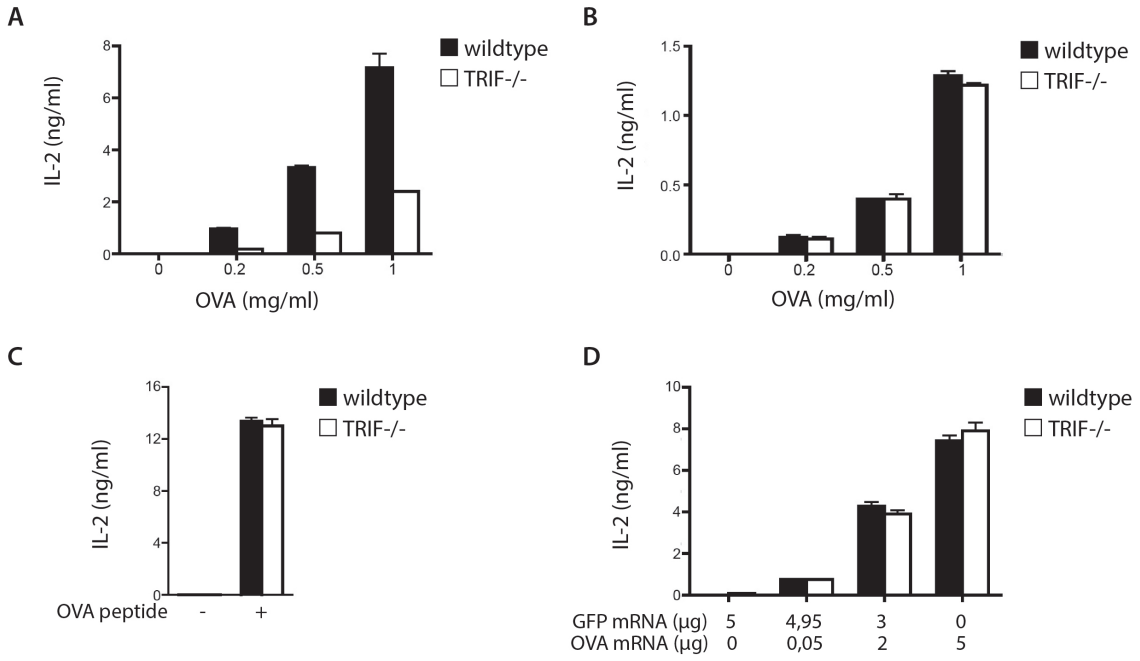

Figure 4.36: ***Cross-presentation is specifically impaired in TRIF-/- DCs*** **A)** Wild-type and TRIF-/- BMDCs were incubated with different OVA-concentrations for 2 hrs and co-cultured with OT-I T-cells overnight. 18 hrs later, the IL-2 concentration in the supernatant was determined by ELISA. **B)** The same as A) with OT-II cells. **C)** The same as A) with 10 nM SIINFEKL peptide instead of OVA-protein. **D)** BMDCs (wild-type or TRIF-/-) were electroporated with mRNA encoding OVA or GFP and were incubated for 2 hrs. Afterwards, theses DCs were co-cultured with OT-I cells overnight and T-cell activation was measured by IL-2 determination. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM.

Additionally, we stained OVA-treated wild-type or TRIF-/- cells with the 25-D1-16 antibody to detect the amount of OVA-peptide loaded MHC-I molecules on the cell surface and analyzed again the cross-presentation efficiency in TRIF-/- cells. This staining confirmed the IL2 ELISA results, proving an impaired cross-presentation in TRIF-/- DCs compared to wild-type DCs. In contrast to this, the staining was equivalent when the processed SIINFEKL peptide was loaded onto the MHC-I molecules at the cell surface, demonstrating that the availability of MHC-I molecules suitable for OVA-presentation was equal on wild-type and TRIF-/- DCs (Fig. 4.37 A). Again the reduction of cross-presentation was not due to different amounts of OVA taken up by these DCs (Fig. 4.37 B), indicating a specific impairment in the cross-presentation machinery.

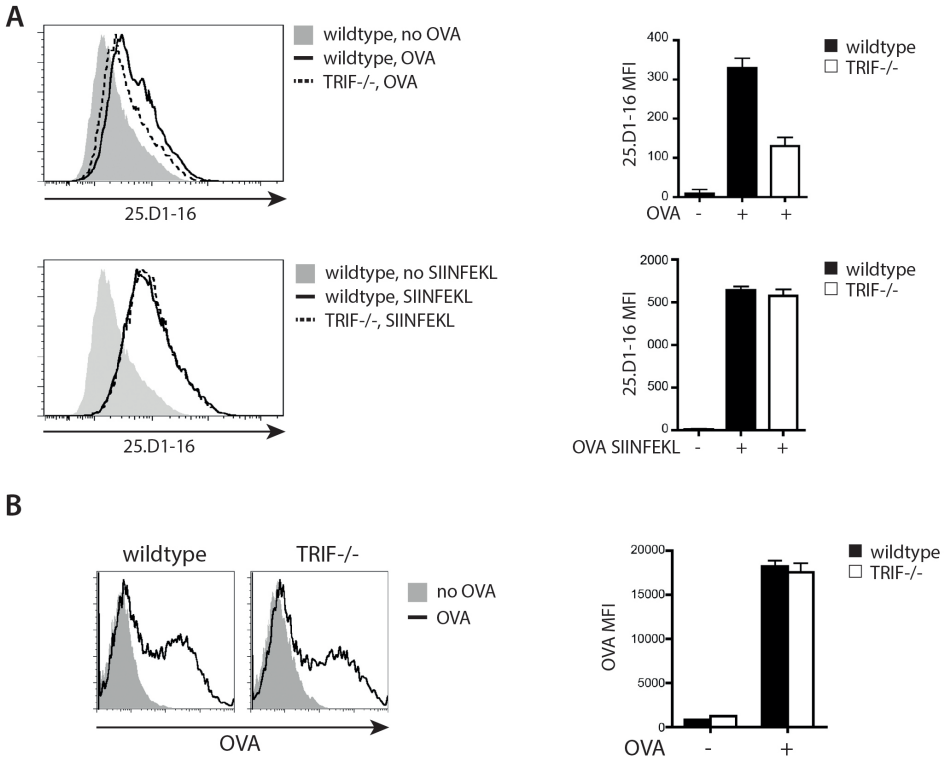

Figure 4.37: ***Cross-presentation dependent display of MHC-I OVA complexes is reduced in TRIF-/- DCs*** **A)** Wild-type and TRIF-/- BMDCs were incubated for 6 hrs in presence of 5 mg/ml OVA. Afterwards, OVA-peptide MHC-I complexes were stained with 25.D1-16 antibody and data were analyzed by flow cytometry. **B)** Wild-type and TRIF-/- BMDCs were incubated with 250 ng/ml fluorophore-labeled OVA for 15 min and analyzed by flow cytometry. All graphs shown represent the results of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

Starting on these results we now investigated whether reduced cross-presentation in the absence of TRIF-signaling might be due to an altered antigen translocation into the cytosol. To test this hypothesis, we again used three different methods to determine the efficiency of antigen translocation into the cytosol. First we fed the antigen OVA to wild-type or TRIF-/- DCs and isolated the cytosolic fraction. Here, we observed a reduction of antigen translocation into the cytosol (Fig. 4.38 A, shown experiment was done by Vera Eulenberg, diploma student), indicating an involvement of TRIF signaling during the antigen translocation. To confirm these results, we fed cytochrome c to wild-type and TRIF-/- cells. In this assay the AnnexinV staining was reduced, when the TRIF-signaling was missing, reflecting a reduced cytochrome c export into the cytosol (Fig. 4.38 B) and verifying the analysis of the cytosolic fraction of OVA-loaded cells.

In agreement with these data, also the β-lactamase assay performed by Dagmar Fehrenschild showed an impaired export efficiency of β-lactamase in TRIF-/- cells (Fig. 4.38 C). By this, we demonstrated that indeed TRIF-/- signaling is required for an efficient antigen translocation into the cytosol.

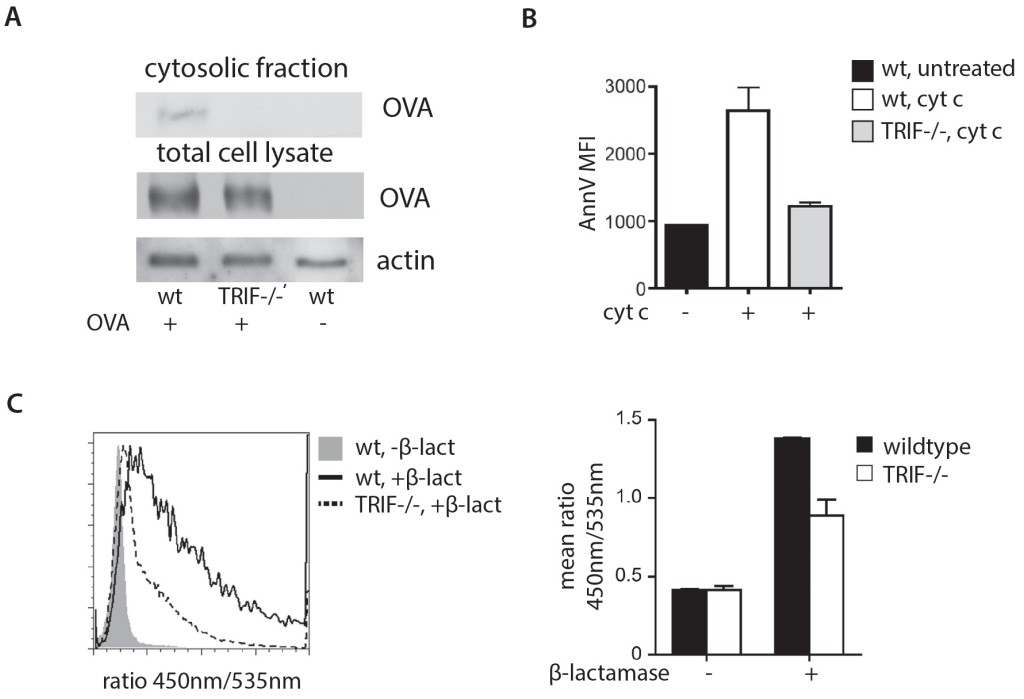

Figure 4.38: ***TRIF signaling is required for efficient antigen export*** **A)** BMDCs of wt or TRIF-/- mice were were loaded with 0.5 mg/ml biotinylated OVA for 45 min in presence of the proteasome inhibitor MG132. Afterwards, the cytosolic fraction was isolated and the exported amount of OVA was determined by western blot. Shown experiment was done by Vera Eulenberg, diploma student. **B)** Wild-type or TRIF-/- BMDCs were treated with 9 mg/ml cytochrome c and 200 ng/ml OVA for 8 hrs. Afterwards, the cells were harvested, stained for AnnexinV and analyzed by flow cytometry. **C)** TRIF-/- BMDCs or control cells were loaded with CCF4 for 1 hr and treated with β-lactamase for another 2 hrs. The cells were harvested and analyzed by flow cytometry. Analysis was done by the ratio of split FRET substrate (emission at 535 nm) to remaining loaded FRET-substrate (emission at 450 nm). The presented β-lactamase data were generated by Dagmar Fehrenschild, bachelor student. All shown graphs are representative results of at least 3 independent experiments. Data are presented as mean $\pm$ SEM. MFI: mean fluorescence intensity.

To analyze, if this impaired antigen trafficking into the cytosol was caused by a missing recruitment of sec61, we transduced wild-type or TRIF-/- DCs with the GFP-sec61β protein and isolated the crude endosomal fraction of these cells after loading them with fluorophore-labeled OVA. Detecting the antigen-positive endosomes by flow cytometry pointed out that indeed sec61 was efficiently recruited only in wild-type DCs, while less co-localization of the antigen-containing compartments and GFP-sec61β was detected in TRIF-/- DCs. Control endosomal flow cytometry of wild-type or TRIF-/- DCs expressing only GFP showed no GFP-signal in the cross-presenting OVA-positive compartments (Fig. 4.39). These data verified the importance of TRIF signaling for the sec61 recruitment towards antigen-containing compartments and subsequently antigen translocation into the cytosol.

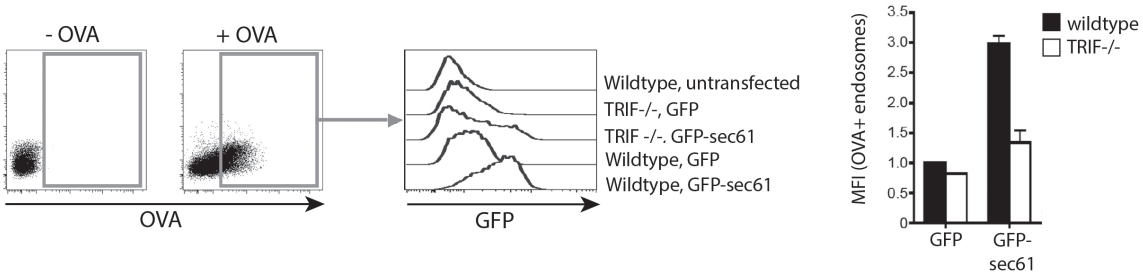

Figure 4.39: ***Sec61 recruitment to antigen-containing compartments is controlled by TRIF-signaling*** Wild-type or TRIF-/- BMDCs were transduced by lentiviruses encoding GFP or GFP-sec61β protein. After additional 3 days of expression the cells were fed with 500 ng/ml fluorophore-labeled OVA for 20 min and chased for another 20 min. To analyze the sec61 recruitment, the cells were harvested, homogenized and investigated by flow cytometry of endosomes. All graphs depict representative results of at least 3 independent experiments. Data are represented as mean ± SEM. MFI: mean fluorescence intensity.

Altogether, the results of this study provided conclusive functional evidence that sec61 is important for antigen export into the cytosol in context of cross-presentation. The presence of sec61 at antigen-containing compartments is thereby essential for antigen translocation and regulated by TRIF-TLR signaling, while it is not significantly influenced by the MyD88 TLR-adaptor protein.

5 Discussion

Based on experiments with the inhibitor ExoA, which impairs the function of the ERAD system [156], a role of the ERAD machinery in cross-presentation has been postulated for a long time [65, 78, 172]. However, the lack of functional data led to an intense and controversial discussion about the mechanisms mediating antigen translocation into the cytosol [173]. During my PhD thesis we were able to provide for the first time a functional evidence that the ERAD machinery participates in the antigen export into the cytosol. In these experiments, we incontrovertibly showed that sec61 is involved in cross-presentation and its function is essential for antigen translocation towards proteasomal degradation and antigen processing. Importantly, the localization of sec61 at the antigen-containing endosomes was required for efficient antigen export. By blocking the recruitment of sec61 to endosomal compartments without affecting its function in the ER, the antigen export was diminished. We were also able to demonstrate that this recruitment is well controlled by a TRIF-dependent TLR signaling.

5.1 Importance of the ERAD machinery for antigen cross-presentation

The molecular mechanisms of cross-presentation have been in the center of interest of immunological science for a long time and several structures have already been reported to be involved [98, 174, 175]. For cross-presentation via the endosome to cytosol pathway, which is propably the most investigated one, several mechanistic aspects have been described. These include the fact that the antigen processing and peptide loading can take place spatially separated from the endogenous MHC-I loading machinery. Also the reimport of peptides into the endosomal cross-presenting compartment via TAP has been convincingly shown in various publications [68, 69, 176]. However, one still very intensively discussed question is how the antigens can pass the membrane barrier the other way around, from the endosomal compartment into the cytosol for proteasomal degradation.
One possibility proposed by some research groups, is that the antigen might leak out of endosomal compartments or simply diffuse into the cytosol because the endosomes might lose their membrane integrity [177, 178, 179]. In line with this it has been shown that a dectamer of the HIV-1 protein Tat can translocate itself and some cargo proteins of different sizes through a phagosomal membrane into the cytosol [178].

Additionally, it was reported that phagosomes containing different pathogens, like *Cryptococcus neoformans*, lose their membrane integrity. Afterwards, proteins can leak out and get access to the cytosolic processing machinery [178]. This more or less "passive mechanisms" might be true for specific pathogens, but it is unlikely that this export-principle can be used for the majority of antigens. Although it has been described that the cellular proteins themselves are able to destabilize the membrane of lysosomal compartments [177], many questions about the diffused antigens remain unanswered, for example the mediation of their further processing in the cytosol and subsequently the MHC-I peptide loading.
However, our results, gained during this thesis, support a second possible antigen-transport mechanism, which uses a channel protein for antigen translocation. In our experiments we found that the inhibition of components of the ERAD machinery showed a strong reduction of antigen cross-presentation efficiency (Figure 4.1 on page 47 and Figure 4.4 on page 49) due to an impaired antigen export into the cytosol (Figure 4.5 on page 50). One of the ERAD inhibitors we analyzed was EeyI, which inhibits the function of the ATPase p97 [164, 165]. Interestingly, this protein has been described before to be involved in the presentation of extracellular antigens on MHC-I [66, 78]. Both, the localization of p97 at the antigen-containing compartment as well as its ATPase function are important for efficient cross-presentation. In this context it is supposed that p97 delivers the required energy for the antigen transfer into the cytosol. This additionally emphasizes that the antigen translocation is rather an active transport event than a passive rupture of the endosomal membrane. The other inhibitor we used was ExoA. This inhibitor is described to interact with the sec61 channel protein and thereby is supposed to block the ERAD associated protein transport through the membrane itself [78, 156]. Also this inhibitor impaired cross-presentation and the antigen translocation into the cytosol (Figure 4.5 on page 50) in our experiments. This is in agreement with results shown by the research group of Prof. Cresswell before [78].
These inhibitor data clearly indicate an involvement of the ERAD machinery in the antigen export events and point out that a channel-based transport mechanism takes place. Because all other antigen presentation pathways remained unaffected (Figure 4.1 on page 47 and Figure 4.4 on page 49), the observed effect on cross-presentation is indeed specific and not due to cytotoxic effects of the inhibitors or caused by stress effects like an unfolded protein response. These stress-responses, which are for example induced by the aggregation of misfolded proteins in the ER, can occur, when the ERAD machinery in the ER is blocked over a longer period of time [180]. Therefore, potential side effects affecting the function of the ERAD machinery in the ER directly or indirectly have to be considered and controlled during all experiments.

Besides of theoretical arguments for the involvement of the ERAD machinery during antigen translocation, like the same transport direction or the use of other ER components during cross-presentation, these inhibitor effects led us to a closer investigation of the role of the ERAD transport machinery in the context of antigen translocation out of endosomal compartments into the cytosol.

5.2 Involvement of the sec61 channel protein during cross-presentation

5.2.1 Sec61 as channel protein for ERAD substrates as well as for antigen translocation

One of the most interesting aspects of the antigen translocation machinery for cross-presentation is, which channel protein is used for the export step. An often mentioned and intensively discussed core protein thereby is sec61 [113, 150, 151, 152, 153], which was, because of the inhibitory effect of the sec61-associated ERAD inhibitor ExoA in our analysis, also the most promising candidate to mediate the antigen translocation in our model system. In line with our data, other research groups have described similar effects of ExoA on cross-presentation. Ackerman *et al.* for example showed in an *in-vitro* system that the export of the antigen out of phagosomal compartments can be impaired by ExoA [78].

One argument often put up against the sec61 channel protein as a candidate for the antigen export as well as for ERAD substrates is its proposed structure. Analyses postulated a pore size of about 5-8 Å, which was criticized to be too small to efficiently transport antigens [181]. However, these analyses have been made for the closed state of the channel protein. An open state was reported to be expanded up to 40-60 Å [182], which would be sufficient to transport unfolded proteins in both directions. Even tightly folded subdomains might fit through this pore size [183].

These presumptions implicate that the antigen needs to be unfolded before the export can take place. And indeed, the requirement of protein unfolding and therefore the participation of different chaperons during the antigen transport through the membrane has been indicated in several studies before. Beside of the chaperon function of p97 during its attendance at the side of antigen export [78, 184], Hsp90 was reported to be involved in antigen translocation [185, 186]. It was also shown that the thioesterase GILT plays a role in the context of cross-presentation by splitting disulfide bounds inside of the antigens [79], emphasizing the importance of peptide unfolding for the export event. These observations are similar to the mechanisms reported from the ERAD system in the ER itself, where misfolded proteins have to be at least partly linearized before they can be exported towards degradation [119, 187].

However, in the ERAD research field itself, it has been postulated during the last years that sec61 can only be used for protein synthesis, but not for retrograde transport [135, 188, 189]. Especially the induction of a channel opening of the sec61 core protein from its luminal side is discussed controversially [190]. Conclusions out of structural analyses predict a model system in which the sec61 pore is blocked by a short helix, named plug. This plug is displaced, when a protein is translated into the ER, but is suggested to block the transport the other way around. Therefore, it is argued that other channel proteins have to take over the transport events for the ERAD machinery.

Nonetheless, so far a direct sec61-excluding evidence is missing and there are many hints that sec61 is important for the ERAD system. One of the substrates, reported for the retrograde transport by sec61 is the cholera toxin, which misuses the ERAD to get into the cytosol. A very interesting finding, arguing for the bidirectional transport possibility of the sec61 channel is the fact that the binding of ribosomes to the sec61 complex for protein translation inhibits the retrograde transport of the cholera toxin [191]. This indicates that a transport directing mechanism exists within the sec61 channel protein. Furthermore, a spacial separation of protein translation and retranslocation would be possible. It was described that sec61 localization is not strictly limited to the ER, but extends into the ERGIC (ER-golgi intermediate compartment) [192]. There, an accumulation of misfolded MHC-I proteins was reported [193]. This suggests that the ERGIC might be a preferred location for the ERAD machinery, while the ER itself is reserved for protein synthesis.

A very recent study in cross-presentation picks up the arguments against sec61 and postulates that this channel protein is not involved in cross-presentation [194]. They used a knock down of sec61α, the major subunit of the sec61 channel complex, as method to analyze its role in cross-presentation. Afterwards, they fed synthetic long peptides to human DCs and observed a moderate, but not significant reduction of the antigen presentation on MHC-I molecules. For knock down control they used western blots and identified a band at 95 kDa as sec61α, which is predicted to be approximately 50 kDa. Additionally, this band at 95 kDa was also affected by siRNA directed against p97 and derlin1, which makes questionable whether the knock down of sec61 was specific and worked efficiently.

In contrast to this, another group did a siRNA approach in the DC2.4 cell line, showing an impaired cross-presentation by the sec61 knock down [172]. Although this result brings sec61 again into the center of interest, important controls like the function of other antigen presentation pathways during sec61 knock down were missing. As mentioned above, these controls are essential to exclude stress effects occurring for example by the unfolded protein response.

To analyze the role of sec61 in cross-presentation more closely, we also knocked down the sec61α subunit in our model and looked at the effects on antigen presentation. After a specific knock down, we observed an impaired cross-presentation (Figure 4.9 on page 54). Importantly, this reduction in antigen presentation was only specific for cross-presentation,

when the cells were analyzed 24 hrs after the knock down and were fixed during the incubation with T-cells. This emphasizes that missing sec61 over a longer period of time indeed affected the general function of the cell (e.g. the MHC-II presentation Figure 4.8 on page 53), perhaps by induced stress effects.
Analysis of the reason for the impaired cross-presentation after the sec61 knock down uncovered that the reduced antigen cross-presentation was indeed caused by a diminished antigen translocation into the cytosol (Figure 4.11 on page 57). In addition we observed the same effect after the knock-down of the sec61γ subunit, which is also important for the function of the sec61 complex (Figure 4.12 on page 58 and Figure 4.13 on page 59), emphasizing the role of sec61 in antigen translocation and cross-presentation.
Since the often used isolation of the cytosolic fractions to analyze antigen export is very sensitive to artifacts by rupture of antigen-containing compartments [71, 195, 196], we did the export analysis with two additional independent assays described by other research groups before (Figure 4.11 on page 57; [64, 197]). This enabled us to ensure the accuracy of the export assay and to confirm the observed effects. Additionally, the usage of three different export assays pointed out that the involvement of sec61 in antigen export is not restricted to the model antigen OVA, but also the export of the two other model antigens (cytochrome c and β-lactamase) depended, at least partly, on sec61.
Nonetheless, it is doubtable that sec61 is the only channel protein involved in the ERAD system as well as in retrograde transport of antigens into the cytosol. So it might depend on the condition of the cell, the uptake mechanism and the substrate itself, which channel protein is involved in antigen export.

5.2.2 Derlin1 knock down has no influence on antigen translocation

One other protein described in the ERAD system as potential candidate to build a channel through the ER membrane is derlin1 [137, 138, 142]. Therefore, derlin1 was also suggested to be involved during antigen export into the cytosol [98, 197] and we analyzed the influence of derlin1 on antigen processing in our used system by siRNA knock down (Figure 4.6 on page 51). However, no effect was observable, although the derlin1 expression was reduced to about 20% of the normal protein level (Figure 4.7 on page 51). These data agree well with the results shown by Menager *et al.*. They also did not detect any influence of derlin1 knock down on cross-presentation [194]. Additionally, the unchanged MHC-II presentation (Figure 4.7 on page 51) indicates that the lack of derlin1 did not induce potential stress effects, which might affect the antigen presentation. This implies that derlin1 either plays a minor role in the recycling of misfolded proteins in the ER during these experiments or that its missing can be compensated by other channel proteins, perhaps derlin2 or derlin3. Having a closer look on antigen-containing endosomes showed no derlin1 localization at these compartments (Figure 4.16 on page 63). These results make it also unlikely that

derlin1 has a function in antigen cross-presentation, which could be compensated by other proteins after derlin1 knock down. In the case that derlin1 might nevertheless be involved in the transport of other antigens than OVA out of endosomal compartments into the cytosol, a separate and strictly regulated recruitment system for derlin1 to antigen-containing endosomes would be required.

5.2.3 Ubiqutin-ligases in the context of cross-presentation

A very interesting and still unanswered question is the influence of ERAD associated E3 ubiquitin ligases in cross-presentation. Besides some functions in substrate recognition for the ERAD system [123], they are required to connect ubiquitin chains to exported misfolded proteins and mark them as substrates for the proteasomal degradation machinery [125, 126]. Therefore, they are essential for the function of the ERAD system. The importance of ubiquitination in context of cross-presentation is largely unknown. In general it was observed that ubiquitinated, but so far undefined, proteins are present on the cytosolic side of antigen-containing compartments [176]. The fact that proteasomal degradation is involved in cross-presentation [44, 80], in combination with the fact that ubiquitination in general is important for proteasomal degradation [34, 36], makes it likely that ubiquitination also plays a role in cross-presentation. Additionally, we recently showed that ubiquitination of the mannose receptor in context of cross-presentation is required for the recruitment of p97 to antigen-containing compartments, which in turn is important for the antigen translocation into the cytosol [66]. This points out that ubiquitination indeed seems to be involved in cross-presentation. Considering the influence of other ERAD components on cross-presentation, it is supposable that ubiquitination of components of the export machinery or of the antigen itself is also mediated by ERAD-associated ubiquitin ligases. Interestingly, ERAD ubiquitin ligases possess, apart from their reactive center in the cytosol, multiple transmembrane domains indicating more functions than the protein ubiquitination alone.

One of the most investigated ER-ubiquitin ligases is Hrd1 [120, 121, 122]. For this ubiquitin ligase also a participation in the ERAD channel system was suggested. So it has been proposed that Hrd1 takes part in the formation of the derlin-dependent core complex [188, 198]. This complex generation would make an involvement of Hrd1 in antigen translocation itself unlikely, because derlin1 was not required for antigen export into the cytosol (Figure 4.7 on page 51). However, the Hrd1 ubiquitin ligase has also been described to be important for the ERAD machinery in many other cases, independent of derlin1. Additionally, it was recently reported that Hrd1 can build a channel for protein translocation by itself [135]. Hence, it would be interesting to see, whether Hrd1 might be involved in antigen translocation or even build an alternative export channel for antigen

cross-presentation. Further experiments and sequential analysis of the export events will be needed to clarify these questions in more detail. Additionally, the potential participation of other known ERAD ubiquitin ligases [124], besides of Hrd1, will be an important aspect for upcoming analyses.

5.3 Recruitment of sec61 towards endosomes is essential for antigen translocation into the cytosol

In several studies antigen cross-presentation is described to be spatial separated from the endogenous MHC-I presentation machinery [62, 98, 199]. Although the complete separation is still under discussion regarding the peptide loading on MHC-I molecules [65, 68], the community agrees that the antigen export takes place at endosomal or phagosomal compartments. In contrast to this, sec61 is a very well described protein with location in the ER and often used as an ER-marker protein. However, for antigen translocation the channel protein needs to be localized in close contact to the antigen itself. Therefore, it was important to clarify, whether sec61 is recruited to the endosomal antigen-containing compartments.

5.3.1 Flow cytometry of endosomes to analyze antigen-containing compartments and sec61 recruitment

Several research groups have already reported the transport of various proteins, which are involved in cross-presentation, from the ER to endosomal compartments (like TAP or MHC-I) [62, 64, 67]. Unfortunately, for the analysis of sec61 recruitment, immunofluorescence microscopy was very difficult to use, because of the very intense signal of sec61 in the ER. Therefore, we decided to use endosomal flow cytometry to get rid of the ER background signal and to more closely analyze the contents of the antigen-containing compartments. This method has already been used to detect the recruitment of p97 to endosomal compartments in the context of cross-presentation [66]. For flow cytometry of endosomes, as described before [157], we carefully opened up the cells by mechanical forces, labeled the targeted proteins extra-endosomal and measured the staining by flow cytometry. This read out is very sensitive and well suitable for the analysis of weak signals at the endosomal compartments.
The function of this method is additionally demonstrated in this work by characterization of the endosomal maturation of antigen-containing compartments in macrophages. The detection of a decreasing number of rab5 molecules, an early endosomal marker,

and an increasing signal of lamp1, a late endosomal and lysosomal marker, at the antigen-containing compartments during the incubation time pointed out that also dynamic processes can be well characterized by flow cytometry of endosomes (Figure 4.14 on page 61).
To investigate the recruitment of the sec61 channel protein, a GFP-sec61β fusion protein was used. In the flow cytometry of endosomes this construct co-localized well with the antigen taken up by the DCs before (Figure 4.15 on page 62). Additionally, to closer characterize the antigen-containing compartment, we stained the crude endosomal fraction of GFP-sec61β expressing DCs for the early endosomal marker rab5 (Figure 4.16 on page 63). Here, a co-localization with sec61 was visible, which is in line with previous studies describing endosomal cross-presentation compartments as structures with stable early endosomal characteristics [69, 70, 71, 200]. Thus, this endosomal analysis revealed that sec61 is indeed localized at the side of the antigens in endosomal compartments and is able to contribute to the cross-presentation machinery. Additionally, we generated in collaboration with Prof. Abraham Koster and Erik Bos electron microscopy pictures, which show a specific sec61-staining at antigen-containing endosomal compartments (data not shown) and confirm our results of endosomal flow cytometry .
In contrast to the sec61 recruitment, derlin1, which was not involved in cross-presentation during our analyses, was not detected at the endosomal compartments (Figure 4.16 on page 63) as already mentioned above. This points out that an unspecific recruitment of sec61 to endosomal compartments does not takes place, like it might occur by fusion events with the ER [82], but the protein acquisition of antigen cross-presentation compartments in general is well controlled and restricted to determined ER-components [64, 67, 73]. One well described example is the ER-localized aminopeptidase ERAP. The ERAP protein is involved in endogenous MHC-I presentation by trimming peptides in the ER, but is not present in endosomal compartments during cross-presentation. It is excluded from antigen-containing compartments [63], whereas TAP is translocalized to cross-presenting structures [64].

5.3.2 Usage of intrabody constructs to modulate cellular functions

To analyze the importance of the specific sec61 recruitment towards antigen-containing compartments for cross-presentation, we planned to inhibit this protein translocation. For this we used an approach with intracellular expressed antibodies (intrabodies) binding to sec61. This enabled us to specifically block the sec61 protein transport and to avoid unspecific side effects, which would occur for example by the use of different inhibitors.
An intrabody is a construct consisting out of the variable parts of an antibody connected by a linker sequence (Figure 4.17 on page 64) and can be expressed within a cell, where it subsequently binds to its target and modulate it in different ways [201, 202]. This system is a very useful tool and becomes, together with *in-vitro* generated antibodies, more and

more interesting for the research community. The evaluated usage of *in-vitro* selected antibodies or intrabodies is due to the quick availability of various antibody genes by phage display for the selection of target-binding constructs [203]. Additionally, the phage display provides the possibilities to find antibodies or intrabodies against difficult targets and to select constructs with specific characteristics [204]. Therefore, this approach is beneficial for many different analyses and several functional assays have been reported during the last years [168, 204, 205]. On the one hand *in-vitro* selected antibodies can be used to target cell surface receptors and modulate their activity, like the B-cell stimulation by an *in-vitro* selected antibody against CD40 [206]. On the other hand intracellular expressed antibody constructs, called the intrabodies, can fulfill a broad range of functions. First, they are very flexible in their location inside of the cell, determined by different localization and retention sequences [201, 207]. Once expressed, the intrabody can for example induce the degradation of the aimed protein, which results in a down-regulation of the target protein [168]. Additionally, intrabodies can modify the intracellular localization and function of proteins [207]. Especially this manipulation of the intracellular trafficking of proteins by intrabody expression was interesting for our studies. In several publications, it was reported that proteins, which are normally transported from the ER to other subcellular compartments can be retained in the ER by intrabodies and thereby are functionally knocked down without directly inducing protein degradation [205, 208, 209]. One example is TLR9, which is normally, after stimulation of the cells occurred, transported from the ER to endosomal compartments containing the TLR9 ligands. This recruitment and subsequently the TLR9-signaling was successfully inhibited by intrabody-mediated ER-retention of the TLR9 molecules [205].

5.3.3 Sec61 recruitment is needed for antigen export into the cytosol

Based on these reports, we generated, in cooperation with the research group of Prof. Dübel, an intrabody binding to a peptide of sec61α with the help of a phage display. The intrabody-binding to the cellular expressed sec61 protein was afterwards verified by immunoprecipitation (Figure 4.18 on page 65). In addition to this, we modified this intrabody to be expressed in the ER and be retained there by a KDEL sequence (Figure 4.19 on page 66) to inhibit the translocation of the sec61 complex towards endosomal compartments. This approach enabled us to specifically analyze the importance of the recruitment of sec61, which has so far not been addressed by the inhibitor or knock down assays done before [78, 172].
Indeed, when we expressed this intrabody in DCs, we detected no sec61 recruitment to endosomal compartments any more (Figure 4.24 on page 71), while TAP as well as calnexin recruitment, which was described by other research groups before [80, 62, 63], were not inhibited by the presence of the sec61α-binding intrabody (Figure 4.25 on page 72). The missing of the sec61 recruitment thereby severely impaired specif-

ically the cross-presentation and inhibited the translocation of various antigens into the cytosol (Figure 4.26 on page 73 and Figure 4.28 on page 75). Importantly, no knock down effects on the expression of the target protein occurred (Figure 4.20 on page 67), which has been described for some intrabodies before [168]. An aspect potentially arguing against the direct involvement of sec61 in cross-presentation would be that the binding of the intrabody to sec61 inhibits the ERAD system and induces stress effects. Alternatively, it might impair protein synthesis of components involved in cross-presentation. To exclude potential stress effects, we first controlled the other presentation pathways (endogenous MHC-I presentation or MHC-II presentation; Figure 4.26 on page 73). These were not affected in the presence of the sec61α-binding intrabodies. To control the function of sec61 in the ER and to exclude that the protein translocation pathway was affected in any kind, we used a tool generated by Grotzke *et al.* [162], namely a HEK-cell line expressing a split Venus protein as reporter. For its function one half of the Venus protein is expressed in the cytosol, while the other half is synthesized into the ER. The ER-expressed segment is modified in a way that it will be glycosylated after translation into the ER and has to be deglycosylated before the export via the ERAD system occurs. Only under these conditions an interaction with the cytosolic half is possible and subsequently a fluorescence signal can be emitted. This analysis showed neither an influence of the sec61α-binding intrabody in protein synthesis nor in the ERAD system itself (Figure 4.21 on page 68). In contrast to this, the inhibition of the ERAD machinery with the sec61-associated inhibitor ExoA or down-regulation of sec61α by siRNA for a longer period of time impaired the Venus-fluorescence, verifying the function of this system. To reinforce this results and to emphasize that the protein translocation by the ERAD system in the ER is unaffected by the sec61α-binding intrabody, we expressed two proteins, which are well known to be degraded by the ERAD system, inside of our DCs [169, 170]. These proteins we fused to the part of the OVA-protein sequence containing the MHC-I epitope SIINFEKL. After protein degradation by the ERAD system, the MHC-I presentation can be used as measurement for the ERAD function. Also this control assay showed no influence of the sec61α-binding intrabody on the function of the ERAD system (Figure 4.22 on page 69).

To additionally exclude that only the synthesis of single proteins, which are not covered by the used control assays, was affected and to definitely prove that indeed only the inhibited recruitment of sec61 was the reason for the reduced antigen translocation into the cytosol, we controlled the effects of the expression of the sec61α-binding intrabody without ER-retention signal. Because this construct is also partly present in the ER (Figure 4.30 on page 77) and is therefore able to interact with sec61, but not to retain it there, it allowed us to reduce the investigation only to the effects of the ER-retention of the sec61

channel protein. Additionally, it excludes side effects by the sec61α-binding intrabody expression itself. This KDEL-missing intrabody showed no reduced antigen translocation or cross-presentation compared to an unspecific control intrabody (Figure 4.31 on page 78) and clearly demonstrates that the intrabody-binding alone has no impairing effect in antigen presentation.
These control experiments clearly point out that the observed reduced cross-presentation was indeed exclusively due to an inhibited sec61 recruitment to endosomal compartments, and emphasize that the translocation of sec61 is needed for an efficient antigen export into the cytosol. Additionally, it demonstrates the intrabody approach as an unique experimental setup to inhibit the recruitment of a protein from the ER to the endosomal compartments with a high specificity and therefore as a very useful tool for further studies in the context of cross-presentation.

5.4 Complexity of protein recruitment and regulatory effects during cross-presentation

Although the intrabody approach clearly demonstrated that sec61 recruitment is essential for antigen cross-presentation, the regulatory mechanisms for the antigen translocation system as well as the recruitment pathway used for important components of this machinery are still unanswered questions, which need to be clarified.

5.4.1 Regulation of the activity of the protein translocation machinery

One important question is, at which steps of the antigen export a regulation of the protein transport is possible. While different control or regulation mechanisms are known for the ERAD machinery at the ER, like the glycosylation structure of target proteins [114] or extending and trimming of the ubiquitin chain of the ERAD substrate [210], it is largely unknown how the export-control is done in context of the cross-presentation machinery. An interesting hint that the antigen export can also be controlled by ubiquitination was recently discovered in our laboratory [66]. We were able showed that the block of polyubiquitination by the monoubiquitin-binding factor TSG101 at the side of antigen export inhibited the recruitment of the ATPase p97, which is needed to deliver the energy for the translocation event. Another potential target for regulation is the sec61 channel protein itself. A very inspiring and puzzling aspect regarding potential regulating factors of sec61 was delivered in the screening for members of the ERAD machinery by Grotzke *et al.* [162]. In this study the sec61α_1 knock down resulted, as expected, in a reduced retrograde transport, whereas sec61α_2 knock down led to an increase of the degradation of exported proteins. These opposing effects might connect sec61α_2 to a

regulatory function of the ERAD machinery. Regardless of its low expression level in DCs under normal conditions (Figure 4.8 on page 53), it remains an open question whether sec61α_2 plays a role in the regulation of antigen processing under certain conditions.

5.4.2 Different recruitment pathways available for the loading machinery of cross-presentation

In contrast to the direct activity control of the antigen translocation machinery, more is known about the recruitment of the cross-presentation machinery to endosomal compartments. Although still many questions remain unanswered, several potential recruitment routes of proteins to endosomal cross-presenting compartments have been postulated. In a recent study a transport pathway for MHC-I molecules was discovered to be essential for cross-presentation [67]. For this, SNARE proteins localized at antigen-containing compartments were used to analyze potential recruitment directions. It was shown that MHC-I molecules, most likely originating from the plasma membrane, were transported into a specialized storage compartment in a rab11a dependent manner. From this compartment the further transport to the cross-presentation compartment was done by SNAP23, which is co-localized with the internalized antigen.
Another transport pathway for the recruitment of ER components to antigen-containing compartments was reported by the research group of Prof. Amigorena [64]. They described that the transport of ER proteins occurs through the ERGIC to the antigen-containing compartment. The ERGIC is a stable sub-compartment of the ER, mainly fulfilling the quality control of newly synthesized proteins [211]. Additionally, it contains parts of the MHC-I complex, which ensures MHC-I loading with high affinity peptides [212]. This transport pathway is mediated by the SNARE protein couple sec22b and syntaxin4 [64]. Because of two aspects, this transport pathway is also very interesting for the recruitment of sec61. First, it was reported by Cebrian *et al.* that the inhibition of this transport system by sec22b knock down impaired the antigen export out of the antigen-containing compartments into the cytosol [64]. One possible reason for this might be a reduced sec61 recruitment to the site of cross-presentation, which is, as shown here in our study, important for efficient antigen translocation into the cytosol. Apart from this, the expression of sec61 within a cell is not completely restricted to the ER, but its presence is extended into the ERGIC, as already mentioned above. Although the exact reason for this expression pattern is still unclear, it would provide an adequate supply of sec61 molecules for the transport event from the ERGIC to the antigen-containing endosomes.
Also several additional proteins regulating the vesicle trafficking have been described to be involved in cross-presentation and ER protein recruitment, like rab27 for the NOX2

translocation [73, 84] or potentially rab14 and syntaxin 6 in the context of the IRAP localization [63, 86]. It remains to be puzzled out, which proteins and recruitment pathways belong to each other and which regulatory mechanisms are used to get a complete and efficiently working cross-presenting compartment.
Next, it was shown in recent studies that endosomal compartments traffic and mature in close relation to the ER. Several contact sites were discovered by high-resolution microscopy pictures [213]. Thus, also this direct contact between endosomes and ER might contribute to the transfer of ER proteins to antigen-containing endosomes for the presentation of extracellular antigens on MHC-I molecules. Considering the fact that only certain proteins are recruited for cross-presentation, also here a strong regulation of the protein translocation would be needed. A possibility therefore would be the aggregation of defined proteins on ER subdomains, which subsequently fuse with the endosomes [214, 215].
During our analyses we showed that the usage of BrefeldinA (BrefA) efficiently inhibited the recruitment of sec61 to the antigen-containing compartments (Figure 4.15 on page 62). The inhibitor BrefA blocks the complete vesicular trafficking from the ER towards the golgi apparatus and the plasma membrane by inducing a retrograde membrane transport to the ER [167]. This result argues for a transport event actively induced by the antigen uptake or other stimuli caused by pathogen contact and favors a vesicular transport pathway. This most likely originates from the ERGIC, which is also destroyed by BrefA. Although our results of the BrefA inhibition indicate a vesicular recruitment mechanism, it still has to be clarified by additional analyses, if and how ER fusion events participate in the cross-presentation machinery.

5.4.3 Importance of TLR signals for the translocation of the cross-presentation machinery

While still many questions about the exact mechanism of ER-protein transport towards antigen-containing compartments remain open, additional regulatory aspects make the analysis of this complex of different mechanisms even more difficult. Also in the case of sec61 translocation it was indicated by the BrefA experiment that the recruitment is not constitutively active, but an induced and regulated event.
Several previous investigations have already reported that endotoxins are important to induce the transport of ER-membranes to endosomal compartments. These stimulations came from many different sources like protozoa, yeast or simple additions of LPS [62, 63, 156]. The studies pointed out that the protein transport pathways for cross-presentation in general are well regulated and not a steady-state system. In our analyses we showed that efficient sec61 recruitment also needed a stimulus by endotoxins. When transferrin was fed to the DCs before sec61 recruitment was analyzed by flow cytometry of endosomes, only a low level of sec61 proteins was visible in transferrin-containing compartments (Figure 4.32 on page 80). Importantly, transferrin is per se endotoxin-free and

is transported into the same compartment as the antigen. Concordant with this, also endosomal compartments containing endotoxin-free antigen (OVA) did not exhibit a high level of sec61 protein compared to endosomal compartments originating from cells fed with endotoxin-containing OVA (Figure 4.32 on page 80).
Closer investigations revealed that a signal mediated via the TRIF pathway induced the transport of sec61 proteins to endosomal compartments and thereby enabled the efficient export of the antigen into the cytosol. Therefore, also the cross-presentation efficiency depended on TRIF signaling and was impaired in TRIF-/- cells (Figure 4.38 on page 86 and Figure 4.36 on page 84). In contrast to this, MyD88 was not required for efficient antigen translocation into the cytosol, although cross-presentation was affected in MyD88-/- DCs (Figure 4.34 on page 82 and Figure 4.33 on page 81). Interestingly, former studies have shown that the recruitment of other components of the MHC-I loading machinery, like the TAP transporter [62], needed efficient MyD88 signaling. In addition to this, it was recently demonstrated that also the MHC-I recruitment is controlled by TLR-MyD88 signaling [67]. This indicates that multiple signaling and protein transport pathways are needed for the composition of functional endosomal cross-presenting compartments.
How these single pathways are connected with each other is not clarified yet, but a very interesting aspect arises from the analysis of TLR4 signaling. It has been well described during the last years that the TLR4 is the only known TLR, which is able to signal MyD88-dependent as well as TRIF-mediated. The activity is controlled by the localization of the receptor. While TLR4 on the plasma membrane signals via the MyD88 adaptor complex, the TRIF-signaling occurs at endosomal compartments [216, 217]. Interestingly, it has been described that the major source of the TLR4 at endosomal compartments is the ERC (endosomal recycling compartment), which is the same compartment described to be the source of MHC-I molecules for cross-presentation. In parallel to the MHC-I molecules, also the TLR4 supply for this compartment is regulated by the rab11a protein. When this protein is missing, the amount of both proteins, MHC-I as well as TLR4, is reduced in the ERC and the recruitment to the antigen-containing compartment is impaired [67, 102]. Additionally, it has been described that the endocytosed TLR4 receptor, besides of antigen co-localization, is also partly present in compartments positive for the transferrin receptor [217], which is a marker of endosomal cross-presenting compartments in APCs [69]. This may point out a two-step recruitment of the cross-presentation machinery. First, TLR4 recognizes LPS at the plasma membrane and the antigen is taken up into the cell. The resulting MyD88 induction leads to the recruitment of components present in the ERC to the antigen-containing compartments, like MHC-I molecules [67]. Additionally, some ER components like TAP are recruited, which was described to be translocated in a MyD88 dependent manner [62]. In parallel to this, TLR4 is recruited from the ERC to the antigen-containing compartment. Inside of these compartments, a second signaling via the TLR4-TRIF pathway is activated and subsequently a second recruitment step from the ER is induced. The missing components like sec61 are thereby translocated, to

mediate the efficient antigen-processing in the endosomal compartments. Whether the recruitment really takes place this way and how it is coordinated, will be addressed in further experiments and will hopefully be clarified during the next years.
In a study of Nair Gupta *et al.,* also first hints were described to understand the mechanistic background of the regulation of protein recruitment during cross-presentation. So it was shown that the phosphorylation of the SNARE protein SNAP23 in an IKK2 dependent manner, which is controlled on TLR-MyD88 signaling, was needed for the MHC-I translocation [67]. Considering the fact that many SNARE proteins are controlled by phosphorylation [218], this might be a good starting point for further analyses of the regulatory effects on protein translocation in the context of cross-presentation.
Additionally, it is known from several different TLRs (e.g. TLR3, TLR7, TLR9) that they are mainly localized in the ER and are recruited to endosomal or lysosomal compartments as soon as a microbial substance is detected [105, 219, 220]. In combination with the results that UNC93B, an important regulator for this TLR recruitment, is involved in cross-presentation efficiency [220], it seems to be possible that parts of the cross-presentation machinery, like sec61, are transported side by side with TLRs to endosomal compartments. Interestingly, it has been described in human DCs that TLR9 recognizes multimeric CpG in endosomes positive for the transferrin receptor, which is also present in endosomal cross-presenting compartments [221]. This indicates that the TLR transport pathway might end up in the same compartments as the MHC-I loading machinery. The fact that many TLRs are only recruited to compartments, which contain their ligands [222, 223], emphasizes again how strictly the protein transport is regulated in the context of antigen detection and processing. Whether a side by side pathway of TLR and cross-presentation components is indeed the case or if these recruitments are independent from each other, remains to be clarified by further analyses.
In contrast to the reports of TLR-dependent protein acquisition, it was also described by Nair Gupta *et. al.* [67] that the sec22b pathway, which has already been mentioned above, is TLR-independent. This contradicts with other observations, like for example the TAP recruitment to antigen-containing compartments. This is described to be sec22b-mediated [64], but in contrary was also shown to depend on a MyD88 stimulus [62].
In the case that some ER proteins, which are involved in cross-presentation, are really translocated in a TLR independent manner, the question arises, what functions they have in a resting DC, missing other molecules, which are important for cross-presentation and recruited in a TLR-dependent manner, like the MHC-I complex at the endosomal compartments [67]. Interestingly, also we observed in our analysis of the antigen-containing endosomes by endosomal flow cytometry a low level of sec61 proteins in absence of endotoxins (Figure 4.32 on page 80). Additionally, we detected some sec61 positive early endosomal compartments in resting DCs missing antigen-load, indicating that a low level of sec61 molecules might be translocated independent of antigen presence or other activating stimuli. This low recruitment might also take place for other components of the cross-presentation

machinery and is, because of its low amount, hard to detect inside of a cell. The use of the endosomal flow cytometry might bring some light into these steady state recruitment mechanisms. Altogether, these aspects of regulating events during cross-presentation need to be addressed more thoroughly in further experiments.
Interestingly, our results show that MHC-II presentation was impaired only by the knock out of the adaptor molecule MyD88, but not by the absence of the TRIF signaling. In agreement to this, TLR-MyD88 signaling was reported to play an important role in phagosome vesicle fusion events and thereby in maturation towards late phagosomes and lysosomes. This is important for MHC-II peptide loading [224], but avoided during cross-presentation. Therefore, next to the regulatory mechanism of cross-presentation, it also needs to be clarified, how the control of MHC-I cross-presentation and MHC-II presentation by TLR signaling interacts with each other and how it is synchronized under different conditions and in various cells. This is another big question, which has to be analyzed in the next years.

5.4.4 Cross-presentation during immunogenic- and non-immunogenic situations

Progressing from the cellular level of cross-presentation to the influence on the organism in the context of health or infection is still very difficult. This is mainly the case, because the importance of cross-presentation in *in-vivo* systems is hard to determine. Especially to distinguish the role of endogenous MHC-I presentation and presentation of extracellular antigens on MHC-I molecules is challenging, since a direct infection and thereby classical presentation on MHC-I molecules can hardly be completely excluded in the analyses of cross-presentation. Additionally, the contributions of both pathways to the immune response largely depend on the type of antigen available for presentation. Nonetheless, more and more evidences are collected, proving a crucial role of cross-presentation for the immune response against various pathogens. Thus, it has been shown for Toxoplasma gondii infections that $CD8^+$ T-cell activation is mainly mediated by cross-presentation of antigens, which were captured by DCs from infected cells in their surrounding [225]. Similar results about the involvement of cross-presentation have been shown in *candida albicans* as well as HSV-1 infections [226, 227]. For HSV-1 it was additionally reported that most likely lymphoid resident DCs play a major role in cross-presentation. These receive the antigen from non-infected migratory DCs originating from the skin. Infected migratory DCs thereby stayed in the peripheral tissue [228]. Interestingly, the peripheral migratory DCs play an important role in cross-presentation during secondary HSV-1 skin infections [226], reflecting the complexity of the antigen presentation system.
Additional to the antigen presentation during infections, also cross-presentation in non-immunogenic situations has been described, often referred to as cross-tolerance. For the central tolerance, the elimination of self recognizing $CD8^+$ T-cells is described to be

done by medullary thymic epithelial cells, which express a broad range of tissue specific antigens [229, 230]. However, they are very poor APCs regarding their expression of MHC-molecules on their surface. Instead of direct self-antigen presentation by these epithelial cells, it has been supposed by several research groups that DCs take over the job of presentation and induction of cross-tolerance [229, 231]. The elucidation of cross-presentation and cross-tolerance mechanisms thereby play also an important role for the understanding of central tolerance.
Also in the peripheral tissue cross-tolerance was indicated in several studies [232, 233, 234]. Considering the results concerning TLR-dependence of the recruitment of at least parts of the cross-presentation machinery, like studies of several research groups (including ourself) have shown [62, 63, 67], raises up the question, how the molecular mechanisms of cross-presentation works and which proteins are involved, when no TLR stimulus is available. As already mentioned above, we were able to show by endosomal flow cytometry a low level of sec61 at endosomal compartments in resting DCs (Figure 4.32 on page 80), indicating that sec61 might also take part during cross-presentation in non-immunogenic situations. Whether sec61 really is important for tolerogenic cross-presentation and which other proteins are involved this mechanism needs to be addressed in further studies.
How important the understanding of the molecular procedure of cross-presentation is, became more and more clear during the last years. A first clinical tool, which already profits of this knowledge, is the cross-presentation-based vaccination. One system of this vaccination method uses the slow maturation of endosomal structures, which contain the antigen. This stability of the antigen-containing compartment is important to ensure a constant supply of antigens for the presentation on MHC-I molecules. During vaccination the inhibited endosomal maturation can be supported by chloroquine, which blocks the acidification of endosomal or phagosomal compartments [235, 236, 237]. This protects the vaccine from degradation, directs it towards MHC-I cross-presentation and thereby potentiates it efficiency.
A second model system for the induction of cross-presentation by vaccination is the targeting of a vaccine to specific receptors on the DC surface, which are known to mediate cross-presentation. For this, mostly receptors which belong to the C-type lectin receptor family like DEC205 [238] or CLEC9A [239], were used as targets. The advantage of this second vaccination strategy is that the induction of both, cross-presentation against infection as well as cross-tolerance to prevent autoimmunity, is possible. Which of these effects is achieved depends on many different factors, but can be influenced for example by the used adjuvants and the formulation of the used vaccine [240, 241] as well as by the binding region of the vector on the targeted receptor [242].
The experimental results of this PhD thesis have clearly demonstrated the involvement of sec61 in antigen export into the cytosol, which might also be a target for further investigation towards therapies. On the one hand, the improvement of cross-presentation dependent vaccinations might be possible by enhancing the recruitment and function of

the antigen export machinery. With the help of special adjuvants this could be done by activating the TLR-TRIF signaling pathway, which is here proven to be important for the efficient sec61 recruitment towards antigen-containing compartments (Figure 4.32 on page 80 and Figure 4.36 on page 84). Additionally, it might also be plausible that pathogens can misuse or circumvent sec61 and other components of the antigen translocation machinery to avoid their degradation and presentation. It is well known for several examples that the ERAD system itself can be manipulated by pathogens, like the herpesviruses, which exploit the ERAD machinery by inducing the degradation of newly synthesized MHC-I molecules and thereby block their presentation [243]. Also Sec61 can be misused, for example by the retrograde transport of the cholera toxin [191]. It remains to decipher possible influences of pathogens on the antigen translocation during cross-presentation. The aim to find possible starting points for clinical interventions as well as to identify the missing links and the regulatory control mechanisms between antigen uptake, antigen export and antigen degradation is the challenge for the next years.

6 Summary

Cross-presentation enables the DCs to load peptides with extracellular origin on MHC-I molecules. One of the major pathways for cross-presentation thereby is the "endosome to cytosol" pathway. For this, the antigen needs to be taken up by the DC and transported to specialized endosomal compartments. Afterwards, the antigen is translocated into the cytosol, processed by proteasomes and in the end loaded on MHC-I molecules either in the same endosomal compartment or in the ER. While many parts of this mechanism have been clarified during the last years, it is still poorly described, how the antigen can pass the membrane barrier of the endosomal compartment to reach the cytosol for proteasomal degradation, which is required for the generation of presentable peptides. Although sec61 has been proposed to mediate this antigen export, its role remained elusive and was discussed controversially.

Here we were able to demonstrate that sec61 is indeed essential for antigen translocation into the cytosol and hence essential for cross-presentation. Additionally, we proved for the first time that the recruitment of sec61 to the side of the antigen, which is localized in endosomal compartments, is required for an efficient antigen translocation into the cytosol and is well regulated by TLR-signaling.

To encounter this, we showed that the presence of a functional ERAD complex is needed for efficient antigen export into the cytosol. Additionally, antigen translocation and cross-presentation were blocked, when sec61 was specifically knocked-down by siRNA, while endogenous MHC-I as well as MHC-II presentation remained unaffected. Importantly, a short incubation time after knock-down and fixation of the cells after antigen uptake were required to avoid unspecific effects due to the down-regulation of sec61.

In addition to this, we were able to prove that sec61 has to be recruited to the side of antigen processing, the endosomal compartments. To verify this and to investigate the recruitment of sec61 more closely we generated a sec61-specific intracellularly expressed antibody (intrabody) to keep sec61 trapped in the ER and to impair its transport towards endosomal compartments. The lack of sec61 in endosomes resulted in a reduced cross-presentation ability of DCs. The impairment of the presentation of extracellular antigens on MHC-I molecules was thereby due to a hindered antigen export into

the cytosol, which was shown by three independent detection assays for different antigens (OVA, cytochrome c and β-lactamase). Decisive for this analysis was the intact function of sec61 in the presence of sec61α-binding intrabodies in the ER.

Finally, we demonstrated that the presence of sec61 in endosomal compartments is not a permanent condition, but the efficient recruitment of sec61 and hence the antigen translocation into the cytosol is a well regulated process and depends on TLR signaling, namely the signaling via the adaptor protein TRIF.

Taken together, we unambiguously proved that the antigen export into the cytosol is mediated by the specific recruitment of sec61 to antigen-containing endosomes and is well controlled by inflammatory signals.

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A Shortcuts

°C	Celsius degrees
β-ME	β-mercaptoethanol
Ab	antibody
ABTS	2,2'-Azino-di-(3-ethylbenzthiazolin)-6-sulfonacid
APC	antigen-presenting cell
ATPase	adenosintriphosphatase
BMDC	bone marrow-derived dendritic cells
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cDNA	‚complementary' DNA produced out of mRNA
CLIP	class-II-associated chain peptide
DAPI	4', 6-Diamidino-2-phenylindol
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
FCS	fetal calf serum
EDTA	ethylenediaminetetraacetic acid
fig.	figure
EEA-1	early endosomal antigen 1
e.g.	exempli gratia (for example)
ELISA	Enzyme-Linked Immunosorbent Assay
ER	endoplasmatic reticulum
ERAD	ER-associated degradation machinery
ERGIC	ER-golgi intermediate compartment
forw.	forward
FRED	Förster resonance energy transfer

GFP	green fluorescence protein
GM-CSF	granulocytes/monocytes-colony stimulating factor
IB	intrabody
hr	hour
H_2O	water
HRP	horseradish peroxidase
IL-2	interleukin 2
IMDM	Iscove's modified Dulbecco's Medium
IP	immunoprecipitation
IRES	internal ribosomal entry site
kB	kilobase-pair
kDa	kilodalton
l	liter
lamp-1	lysosome associated membrane protein 1
LPS	lipopolysaccharide
LTR	long terminal repeats
M	molar
Mϕ	macrophage
MES	2-(N-Morpholino)ethansulfon acid
MFI	mean fluorescence intensity
MHC-I/II	major histocompatibility complex
min	minute
ml	milliliter
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response gene
NADPH	nicotinamide adenine dinucleotide phosphate
NEM	N-Ethylmaleinimid
NOX2	NADPH oxidase 2
OT-I cells	$CD8^+$-T-cells recognizing OVA-peptide SIINFEKL loaded on MHC-I
OT-II cells	$CD4^+$-T-cells recognizing OVA-peptide ISQAVHAAHAEINEAGR loaded on MHC-II
OVA	ovalbumin
PAMP	pathogen associated molecular patterns

PBS	phosphate-buffered solution
PCR	polymerase-chain reaction
PDI	protein disulfide isomerase
PLP	phosphate lyse-buffer
PRR	pattern recognition receptor
rag1	recombinase activating gene 1
rab5	Ras-related protein
rcf	relative centrifugal force
rev.	reverse
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RRE	rev responsive element
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
SIINFEKL	OVA peptide 257-264
siRNA	short interfering RNA
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
TAE	tris-acetat-EDTA
TAP	transporter associated with antigen processing
TCR	T-cell receptor
TEMED	tetramethylethylendiamin
TfR	transferrin receptor
TGN	trans-golgi network
TLR	Toll-like-receptor
TM-domain	transmembrane domain
TRIF	Toll/IL-1 receptor, domain-containing adaptor inducing IFN-ß
VSV-G	vesicular stomatitis virus glycoprotein
w/o	without
WB	western blot
wt	wild-type

B Declaration of authorship

Hereby I declare that this thesis,

Sec61 mediates antigen translocation into the cytosol for cross-presentation

and the work presented in it is my own and has been generated by me as the result of my own original research. Where the thesis is based on work done by myself jointly with others, I have made clear what exactly was done by others and what I have contributed myself.

Bonn

Signature / date

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D Publications

Mannose Receptor poly-ubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation
Zehner Matthias, Chasan Achmet Imam, Schuette Verena, Embgenbroich Maria, Quast Thomas, Kolanus Waldemar, Burgdorf Sven
Proc Natl Acad Sci 2011

Cross-presentation: how to get there - or how to get the ER.
Kreer Christoph*, Rauen Judith*, ***Zehner Matthias****, Burgdorf Sven
**** These authors contributed equally***
Front Immunol. 2011

Intra-endosomal flow cytometry as a novel approach to analyze the protein content of antigen-loaded endosomes
Zehner Matthias, Imam Chasan Achmet, Rauen Judith, Embgenbroich Maria, Haas Albert, Kurts Christian, Endl Elmar, Beyer Marc and Burgdorf Sven
Eur J Immunol. 2012

Regulation of antigen transport into the cytosol for cross-presentation by ubiquitination of the mannose receptor.
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Endosomal Sec61 mediates antigen translocation in the cytosol for cross-presentation
Zehner Matthias, Andrea L. Marschall, Erik Bos, Jan-Gero Schloetel, Christoph Kreer, Dagmar Fehrenschild, Andreas Limmer, Ferry Ossendorp, Thorsten Lang, Abraham J. Koster, Stefan Dübel, Sven Burgdorf
Immunity 2015